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Testing comparability of existing and innovative bioassays for water quality assessment

A European-wide exercise

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Executive summary

The JRC led a consortium of seventeen research Institutes from eleven countries in EU and associated countries to evaluate the suitability of the current paradigm in environmental risk assessment that considers the risk of single chemicals for assessing water quality. Combined effects of chemical mixtures of concern were measured on different aquatic organisms and different levels of biological organisation using existing and innovative bioassays.

Policy context

Aquatic organisms in most European surface waters were exposed to many chemical pollutants simultaneously.

However, the current paradigm in water quality assessment under the Water Framework Directive (WFD) still considers the effects of single substances instead of evaluating the combined action of environmentally relevant mixtures.

The potential effects of combinations of chemicals are equally relevant to the risk assessment of consumer products and of drinking water to humans.

Key conclusions

We could show that exposure to mixtures of dissimilarly acting substances at concentrations considered environmentally acceptable can exert significant effects on biota. Therefore, chemical monitoring of a few substances may be insufficient to assess the quality status of water impacted by complex anthropogenic mixtures.

The present study highlights an urgent need to revise tools and paradigms used to assess the safety of chemicals to the environment. Bioassays as part of a multi-tier approach to water quality monitoring can fill the gap between chemical and ecological assessments for a more holistic characterisation of water quality.

Considering a potential future revision of the WFD, it is timely to introduce the issue of risk posed by mixtures of pollutants into the discussion table and find innovative ways to assess water quality in a more holistic way than the mere assessment of biological and chemical indicators for ecological and chemical status respectively.

Main findings

Mixtures of 14 or 19 chemicals of concern for the contamination of surface waters, have been produced as reference materials and tested using biological-based assays (bioassays). Each compound was present at its safety concentration limit according to European legislation, the environmental quality standard (EQS). The bioassays covered the most relevant ecotoxicological endpoints and included OECD-validated and non-validated methods. The mixtures included several classes of chemicals, such as pesticides, pharmaceutical compounds and different industrial products.

The results from this exercise showed a general comparability between different bioassays targeting the same biological endpoint and classes of substances. The mixtures of chemicals at concentrations considered to be safe under the current legislation originated effects in some bioassays. These include changes in the algal-bacterial composition in a

marine microcosm, effects on immobilisation in *Daphnia magna*, fish embryo toxicity and effects on frog embryo development. Additionally, increased expression on reporter genes linked with oxidative stress was observed in both *Caenorhabditis elegans* and *Escherichia coli*. Estrogenic receptor-binding assays have detected estrogenic compounds in the mixture close to EQS values. The results indicate the suitability of many of the methods as a first toxicological screening of water samples to facilitate the water monitoring management.

Related and future JRC work

The second EU-wide exercise has been launched to assess a practical employment (application) of described methods to environmental samples and determine the water quality profile by comparing the effects to the reference material. Furthermore this exercise will explore additional compositions of reference materials. Particular focus will be given to detection of estrogenicity effects of the most potent substances at very low (regulatory) concentrations and of other substances in the mixture when estrogenic hormones are not present. In addition, establishment of assay detection limit and identification of additive effects of similarly acting endrocrine disrupting compounds will be tested.

1. INTRODUCTION

The Water Framework Directive 2000/60/EC (WFD) has established a strategy for water protection that included specific measures for pollution control to achieve good ecological and chemical status at European level. Good chemical status was defined in terms of compliance with European environmental quality standards (EQS) for substances of concern, to make sure that the concentrations found in the environment are below a safety limit, not causing any harmful effects to or via the aquatic environment. The selection of substances for regulation has been made for those of European Union (EU)-wide concern (the priority substances that should be compliant with EQS) and those substances of national or local concern (river basin specific pollutants, which should be compliant with national EQS).

Subsequently, Directive 2008/105/EC (EQSD) has set the EQS values of the WFD for priority substances or priority hazardous substances (those of higher concern) in surface waters (river, lake, transitional and coastal). EQS values for annual average (AA) or maximum allowable concentrations (MAC) have been derived to protect against long-term exposure or short-term peak concentrations, respectively. Recently, Directive 2013/39/EU, amending the WFD and EQSD, modified the list of priority substances by identifying new substances for priority action at Union level, setting EQS for those newly identified substances and revised the EQS for some existing substances in line with scientific progress.

The EQS derivation uses a framework methodology (CIS No. 27, 2018) that considers all relevant and reliable information regarding (eco) toxicological effects of single chemicals.

For compliance checking under the WFD, chemical monitoring is essential for determining chemical status of a water body by comparing measured concentrations of a substance with its EQS. For technical and economic reasons, there is a tendency to restrict the chemical analysis to already regulated substances that are known to pose a threat to or *via* the aquatic environment. However, environmental samples are usually very complex, with inflows from agriculture, industry, medical facilities, household waste and others. Therefore, hundreds of different substances may coexist in place and time, even if most are present at very small concentrations.

Strikingly, the effects from the combined action of co-occurring pollutants are not taken into account when assessing water quality. So far, it has been assumed that the safety factors applied in EQS derivation can cover the combined action of pollutants in most situations. However, there has been a growing concern from the public regarding this issue. A recent communication from the European Commission on combination effects of chemicals (COM 2012-252) acknowledges that the potentially toxic effects of co-occurring chemicals in food, water, air and manufactured products are rarely examined, despite the strict limits that are set for the amounts of particular chemicals. It further demands a higher effort to ensure that the risks associated with chemical mixtures are properly understood and assessed.

It is timely to start investigating for more holistic approaches in water quality assessment by testing combination effects from chemical mixtures. Such approaches could eventually fill the information gap between the current ecological and chemical monitoring.

Bioassays offer the possibilities to monitor the overall response from co-exposure to multiple, bioavailable chemicals that co-exist in a water sample. These assays may target different levels of biological organisation, such as community, population, individual and/or sub-organism levels (Wernersson et al. 2015).

One application of biossays could be the initial screening of water samples regarding the main ecotoxicogical endpoints, to provide guidance on which classes of chemicals to analyse subsequently or to concentrate and restrict the chemical analysis in areas of

concern. If such a battery of tests could be implemented, it would allow a more cost-effective water monitoring management.

So far there are only a few standardised OECD methods that are able to give toxicological information on a small number of endpoints, and even few information is available when it comes to the application of those methods to environmental samples. Given the ever increasing research in the field and published bioassays under different stages of development, and because different bioassays are rarely tested on similar field samples, there is limited information on the comparability or overlap of different bioassays, inter-laboratory studies, etc.

To aid a better understanding on the state-of-the art of existing bioassays for the assessment of complex mixtures of chemicals as those found in the environment, a scientific expert meeting was held at the JRC on 19 January 2012, in Ispra, Italy. The participants were all European scientific experts in the fields of ecotoxicology, ecology, environmental genomics and analytical chemistry. The first objective of the meeting was the identification of the main challenges posed by complex mixtures of chemical pollutants to the monitoring of water quality in Europe, under the implementation of EU legislation such as the WFD, the Marine Strategy Framework Directive and the Waste Water Directive. The second objective was to establish a consortium of labs in Europe to address those challenges particularly whether and how the existing and innovative bioassay could contribute to the assessment of water quality.

This report describes the outcome of the EU-wide exercise organised by the JRC with the collaboration and effort of 17 partner laboratories from eleven EU and EU-associated countries. For this exercise, a chemical mixture with chemicals at safety limit concentrations (EQS) was prepared, distributed to each partner and tested by using a total of 32 different bioassays to assess the potential combined toxicity effects of the mixtures.

2. INITIAL WORKSHOP AND STUDY DESIGN

2.1 Selection of chemical mixtures

A first exercise was agreed for the inter-laboratory analysis of complex artificial mixtures of chemicals.

Substances of priority concern were selected taking into account their prevalence in European surface waters, their known effects and a wide representation of different chemical groups. Two chemical mixtures were produced, one containing 14 substances (Mix14) and the other containing 19 substances (Mix19). The chemicals included in Mix14 are listed in Table 1 and included the priority substances atrazine, diuron, isoproturon and simazine (photosystem II inhibiting herbicides), benzo[a]pyrene (BaP) and fluoranthene (polycyclic aromatic hydrocarbons), cadmium and nickel (metals), DEHP (plasticizer), 4-nonylphenol (surfactant), chlorphenvinphos and chlorpyrifos (insecticides), and 17β-estradiol (naturally occurring estrogen) and diclofenac (pain killer) which have been included in the first Watch List (Carvalho et al. 2015). The concentration equivalent to the EQS value for each of these substances was chosen in order to assess the applicability of the methods to concentrations relevant in actual water samples. In addition, for many of the bioassays, Mix14 was tested at additional concentrations.

In addition to the substances listed above, Mix19 contained five emerging pollutants for which there is a growing concern: bisphenol A (used for the production of plastics and food packaging products), carbamazepine (prescription medicine), sulfamethoxazole (a sulfonamide bacteriostatic antibiotic), triclosan (an antibacterial and antifungal agent) and DEET (an insect repellent), all at concentrations corresponding to their EQS value under river basin management plans (Table 1).

Table 1. Chemical composition of the final exposure mixtures used in the EU-wide exercise.

Substance	CAS[a]	Use	Mode of action and reported effects	AA-EQS*	Mix14* 1×EQS	Mix19* 1×EQS
Atrazine	1912-24-9	Herbicide	Photosystem II inhibitor	0.6[b]	0.6	0.6
Benzo[a]pyrene (BaP)	50-32-8	By-product of incomplete combustion of organic material	Intercalation in DNA causing mutagenesis, carcinogenesis	0.00017[b]	0.00017	0.00017
Cadmium (Cd)	7440-43-9	Industrial by-product: used in metal plating and to make pigments, batteries, and plastics. Insecticide	Indirect formation of reactive oxygen species depletion of glutathione, lipid peroxidation	0.08[b]	0.08	0.08
Chlorfenvinphos	470-90-6	Insecticide	Inhibition of cholinesterase activity	0.1[b]	0.1	0.1
Chlorpyrifos	2921-88-2	Insecticide	Inhibition of cholinesterase activity	0.03[b]	0.03	0.03
Bis(2-ethylhexyl) phthalate (DEHP)	117-81-7	Plasticizer	DNA damage, carcinogenicity	1.3[c]	1.3	1.3
Diclofenac	15307-79-6	Pharmaceutical pain killer: non-steroidal anti-inflammatory drug (NSAID)	Can cause adverse hepatic effects in certain organisms	0.1[c]	0.1	0.1
Diuron	330-54-1	Herbicide	Photosystem II inhibitor	0.2[b]	0.2	0.2
17ß-estradiol (E2)	50-28-2	Natural estrogen	Natural estrogen	0.0004[c]	0.0004	0.0004
Fluoranthene	206-44-0	Product of incomplete combustion	Causes mutagenesis, carcinogenesis	0.0063[b]	0.0063	0.0063

Isoproturon	34123-59-6	Herbicide	Photosystem II inhibitor	0.3[b]	0.3	0.3
Nickel (Ni)	7440-02-0	Industry, preparation of alloys	Depletion of glutathione levels, binds to sulfhydryl groups of proteins, carcinogenicity	4[b]	4	4
4-nonylphenol	25154-52-3	Mostly used for the production of surfactants (nonylphenol ethoxylates)	Endocrine disruptor	0.3[b]	0.3	0.3
Simazine	122-34-9	Herbicide	Photosystem II inhibitor	1[b]	1	1
Carbamazepine	298-46-4	Pharmaceutical (anti-epileptic, mood-stabilizing drug)	Teratogenicity	0.5[d]	-	0.5
Sulfamethoxazole	723-46-4	Pharmaceutical (antibiotic)	Interferes with folic acid synthesis	0.6[d]	-	0.6
Triclosan (Irgasan)	3380-34-5	Anti-bacterial and antifungal agent used in cosmetics and detergents	Inhibition of cellular efflux pumps	0.02[d]	-	0.02
N,*N*-diethyl-*meta*-toluamide (DEET)	134-62-3	Insect repellent	Affects insect odorant receptors, inhibits cholinesterase activity (nervous system)	41[d]	-	41
Bisphenol A (BPA)	80-05-7	Plasticizer	ER agonist	1.5[d]	-	1.5

[a] Chemical Abstracts Service
[b] According to European Directive 2013/39/EU
[c] From COM 2011-876
[d] Proposal from Ecotox Centre, Switzerland
*Values reported as µg/L

2.2. Selection of bioassays

Chemical substances released into aquatic environments may affect different organisms, cells and molecular targets, may be more or less bioavailable and partition differently between the water and sediment phases, depending on their structure, solubility and stability. Substances with similar chemical properties may share the same mode of action (MoA) and if co-existing in the same water body, their effect to organisms may be additive. There are also potential synergistic or antagonistic effects that may occur for co-existing substances that compete for binding to the same molecular targets.

In this exercise, diverse bioassays have been proposed in order to assess the effect of the chemical mixtures in a wide-range of biological endpoints and organisms from different trophic levels. These included bioassays measuring cytotoxicity, embryo toxicity, teratogenicity, estrogenic activity, metal toxicity, oxidative stress, among others (Table 2). Additionally, some of the methods have been performed by more than one group. It is important to note that there was no standard operating procedure shared across laboratories assessing the same biological endpoints, and therefore there are clear limitations in extrapolating the consistency of the results.

Table 2. List of bioassays and partner laboratories in the EU-wide exercise.

Organism and/or test \ Partner Laboratory[a]	JRC	EAWAG (CH)	ECOTOX (CH)	RECETOX (CZ)	DTU (DK)	INERIS (F)	HUJI (IL)	ISPRA (IT)	UNIPMN (IT)	NTNU (N)	NIFES (N)	ORU (S)	MBSS (SLO)	AESD King's College (UK)	BOKU (A)	DNS King's College (UK)	BHAM (UK)
Microcosmos in marine water Bacteria production and pigment concentration													X				
Vibrio fischeri - Microtox® EN ISO 11348-3 Inhibition of bioluminescence			X	X				X	X								
Daphnia magna EN ISO 6341 Acute immobilisation				X	X			X									
Daphnia magna CSN ISO 10706 Reproduction test				X													
Pseudokirchneriella subcapitata ISO 8692 Growth inhibition			X	X	X			X	X								
Pseudokirchneriella subcapitata Inhibition of photosysthesis			X														
Chlamydomonas reinhardtii Growth inhibition			X														

Partner Laboratory[a] / Organism and/or test	JRC	EAWAG (CH)	ECOTOX (CH)	RECETOX (CZ)	DTU (DK)	INERIS (F)	HUJI (IL)	ISPRA (IT)	UNIPMN (IT)	NTNU (N)	NIFES (N)	ORU (S)	MBSS (SLO)	AESD King's College (UK)	BOKU (A)	DNS King's College (UK)	BHAM (UK)
Chlamydomonas reinhardtii Inhibition of photosysthesis			X														
Thalassiosira pseudonana Growth inhibition	X																
Saccharomyces cerevisiae Transgenic fluorescent yeast strains for acute toxicity and mutagenicity	X																
Caenorhabditis elegans Growth, lipid accumulation, pharyngeal pumping, movement														X			
Dictyostelium discoideum Mortality, reproduction, lysosomal membrane stability									X								
Xenopus laevis ASTM E 1439-98 Frog embryo teratogenesis assay (FETAX)				X													
Danio rerio EN ISO 15088 Fish embryo toxicity (FET)				X													

Partner Laboratory[a] / Organism and/or test	JRC	EAWAG (CH)	ECOTOX (CH)	RECETOX (CZ)	DTU (DK)	INERIS (F)	HUJI (IL)	ISPRA (IT)	UNIPMN (IT)	NTNU (N)	NIFES (N)	ORU (S)	MBSS (SLO)	AESD King's College (UK)	BOKU (A)	DNS King's College (UK)	BHAM (UK)
Molecular biomarkers																	
Escherichia coli (bacterium) Bioluminescent reporters for stress response							X										
Caenorhabditis elegans (nematode) Dual-fluorescent transgenic organisms for stress response														X			
Saccharomyces cerevisiae (yeast) Reporter genes for genotoxicity and acute toxicity	X																
HeLa, LMH and ZFL cell lines Genes expression analysis of several biomarkers												X					
Ex vivo																	
Gasterosteus aculeatus Impact on splenic leucocyte immune activities						X											
Juvenile Atlantic salmon *(Salmo salar)* xCELLigence system primary cultures of hepatocytes											X						

Partner Laboratory[a] / Organism and/or test	JRC	EAWAG (CH)	ECOTOX (CH)	RECETOX (CZ)	DTU (DK)	INERIS (F)	HUJI (IL)	ISPRA (IT)	UNIPMN (IT)	NTNU (N)	NIFES (N)	ORU (S)	MBSS (SLO)	AESD King's College (UK)	BOKU (A)	DNS King's College (UK)	BHAM (UK)
***In vitro* toxicity tests**																	
MTT assay RTG-2, RPTEC/TERT1, HUVEC/TERT, HepG2 and MCF7								X							X		
PLHC-1 cells 7-ethoxyresorufin-O-deethylase (EROD) activity						X											
Estrogenreceptor (ER) binding assay (estrogenic activity)																	
Yeast estrogenic screen (YES)			X														
ER-CALUX®			X														
ER-binding/activation in MELN cells						X											
EASZY transgenic *cyp19a1b*-GFP zebrafish embryos						X											
Binding to the recombinant human ERα	X																
Estrogenic activity – other assays																	
Atlantic salmon (*Salmo salar)*										X							

Partner Laboratory[a] / Organism and/or test	JRC	EAWAG (CH)	ECOTOX (CH)	RECETOX (CZ)	DTU (DK)	INERIS (F)	HUJI (IL)	ISPRA (IT)	UNIPMN (IT)	NTNU (N)	NIFES (N)	ORU (S)	MBSS (SLO)	AESD King's College (UK)	BOKU (A)	DNS King's College (UK)	BHAM (UK)
Molecular biomarkers Zrp, ERα and Vtg																	
ERα, ERβ gene expression in HeLa cells												X					
Other receptor-binding assays																	
Androgen Receptor (AR) - CALUX®			X														
Peroxisome proliferator-activated receptor ɣ2-like (PPAR)-CALUX®			X														
Pregnane X receptor *(*PXR*)*			X											X			
Aryl hydrocarbon receptor *(*AhR*)*				X										X			
Transcriptomics																	
Oncorhynchus mykiss (rainbow trout gill cell culture system)																X	
Daphnia magna																	X
Thalassiosira pseudonana	X																
Chlamydomonas reinhardtii		X															

[a] The following abbreviations have been used for the different partner affiliations, in alphabetical order:

AESD, Analytical and Environmental Sciences Division, King's College London, UK

BHAM, University of Birmingham, UK

BOKU, University of Natural Resources and Life Sciences, Vienna

DNS, Department of Nutritional Sciences, King's College London, UK

DTU, Technical University of Denmark (Department of Environmental Engineering), Kgs Lyngby, Denmark

Eawag, Swiss Federal Institute for Environmental Science and Technology / ETH

ECOTOX, Oekotoxzentrum/Eawag, Dübendorf, Switzerland

HUJI, The Hebrew University of Jerusalem, Israel

INERIS, National Institute for Environmental Technology and Hazards, Verneuil en Halatte, France

ISPRA, Istituto Superiore per la Protezione e la Ricerca Ambientale (ISPRA), Rome, Italy

JRC, European Commission - Joint Research Centre

MBSS, Marine Biological Station Piran - National Institute of Biology, Slovenia

NIFES, National Institute of Nutrition and Seafood Research, Bergen, Norway

NTNU, Norwegian University of Science & Technology (NTNU), Trondheim, NORWAY

ORU, Life Science Center, Örebro University, Sweden

RECETOX, Research Centre for Toxic Compounds in the Environment, Masaryk University, Brno, Czech Republic

UNIPMN, Università del Piemonte Orientale, Alessandria, Italy

3. CHEMICAL MIXTURES

3.1 Chemical mixtures as reference materials

Due to the fact that the different proposed bioassays usually involve different methods for exposure (e.g. exposure to raw water samples, extraction, concentration), it was decided to produce the chemical mixtures as concentrated materials to maximise the possible applications. The mixtures Mix14 and Mix19 were thus produced as 1000-fold concentrated materials with respect to the final concentrations used in the bioassays (Table 3). Additionally, Mix14 was also produced as 10000-fold concentrated material to broaden the tested concentration range of this mixture.

The reproducibility in the composition of the exposure mixtures in all laboratories was a major concern to allow minimal comparability of the data. For this reason, the concentrated mixtures have been produced as reference materials by the reference laboratory Istituto Superiore per la Protezione e la Ricerca Ambientale, in Rome, Italy.

Reference Materials have been dispatched in dry ice to all partner laboratories by express courier. The laboratories were advised to keep the organic reference materials (ISPRA RM040-042) at the reference temperature (-20ºC), and keep the inorganic reference materials (ISPRA RM043-44) at 4ºC until reconstitution.

3.1.1 Stability study of reference materials

The organic compounds were prepared in methanol. This solvent was selected because of the existing data for solubility of the different compounds at the chosen concentrations in the stock mixtures, and the relatively low toxicity of this solvent in most biological systems at the expected final concentration. Additionaly, the two metals (cadmium and nickel) were prepared in water containing 2% nitric acid.

The procedure for the production of the chemical mixtures as reference materials is summarised in Figure 1. An initial assessment of the solubility of each organic compound in the selected solvent methanol was performed over a period of two months.

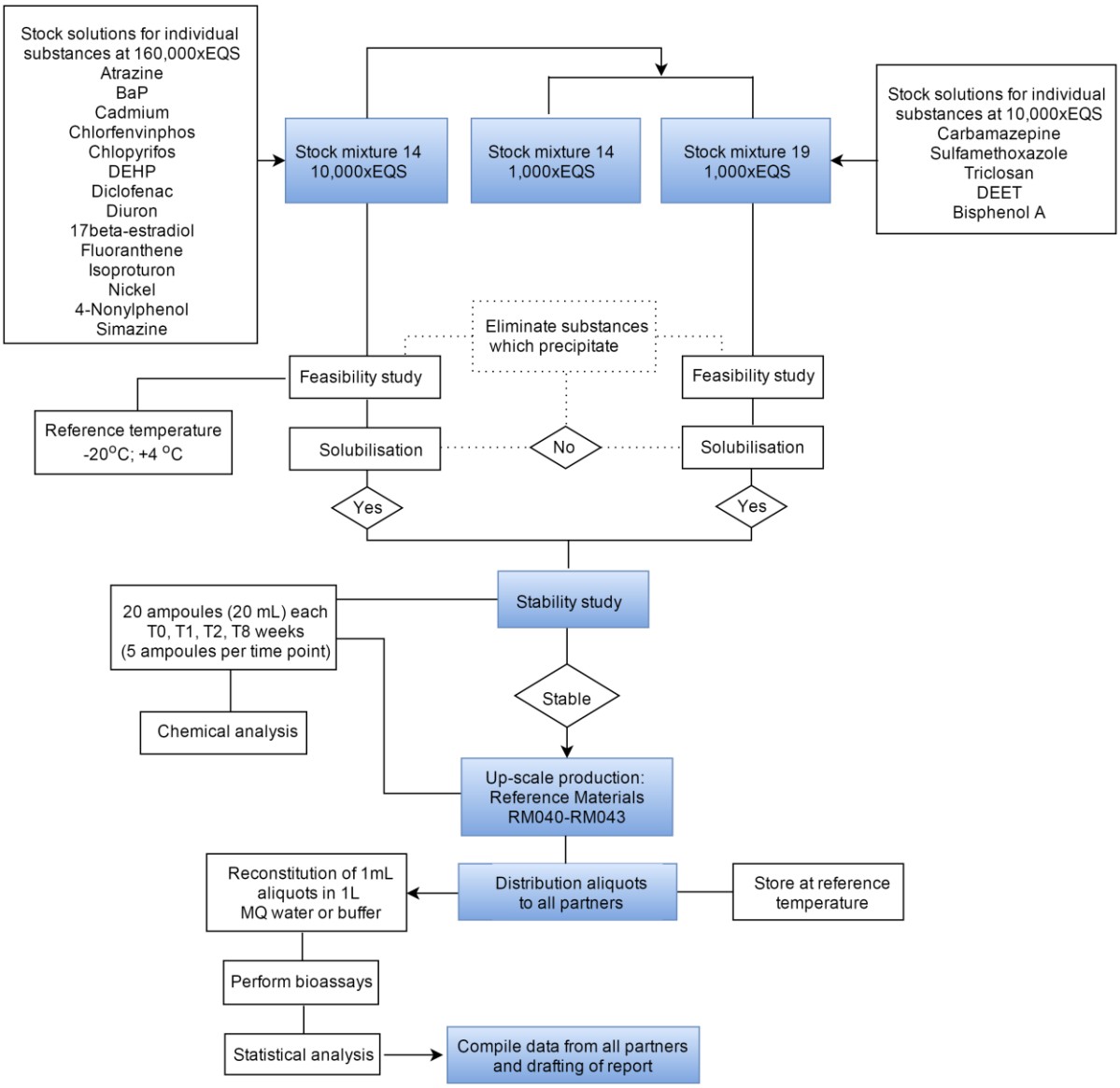


Figure 1. Scheme for the production of the chemical mixtures as reference materials. Concentrated stock solutions for each individual substance have been produced using methanol as the solvent for the organic substances and 2% nitric acid in water as the solvent for the metals. Substances were mixed to produce concentrated stock mixtures at the concentrations indicated in the scheme (as fold EQS) and a feasibility study was made to verify the solubilisation of all the substances in the mixtures followed by a verification of the stability over time at the storage temperature. Once solubility and stability were verified, reference materials were produced as described in Table 3.

Table 3. Concentrated reference materials.

Reference Organic Mixture for preparation of Mix14	Concentration of single chemicals in the mixture	Solvent
ISPRA RM040 (12 organic compounds)	10 000 × EQS	Methanol
ISPRA RM041 (12 organic compounds)	1 000 × EQS	Methanol
Reference Organic Mixture for preparation of Mix19	**Concentration of single chemicals in the mixture**	**Solvent**
ISPRA RM042 (17 organic compounds)	1 000 × EQS	Methanol
Reference Inorganic Mixture	**Concentration of single chemicals in the mixture**	**Solvent**
ISPRA RM043 (2 inorganic compounds)	10 000 × EQS	2% nitric acid in water
ISPRA RM044 (2 inorganic compounds)	1 000 × EQS	2% nitric acid in water

A short-term stability study on the candidate reference materials was carried out to take into account any improper shipment and storage conditions during the intercomparison exercise.

The stability experiment was designed according to an isochronous study. This approach minimises the variations in analytical response as a function of time since all sample analyses are done at the end of the study (ISO Guide 35, 2006; Lamberty et al. 1998; Linsinger et al. 2001), by dividing results obtained on samples stored at a given temperature by results obtained on samples stored at a reference temperature. The results are expressed as the ratio between the concentrations of samples stored at a temperature T and a reference temperature T_0. The reference temperatures were set at -20±4°C and 4±4°C (where degradation and instability was supposed not to occur) for the organic reference materials (i.e. ISPRA RM040, ISPRA RM041 and ISPRA RM042, described in Table 3) and the inorganic reference materials (i.e. ISPRA RM043 and ISPRA RM044), respectively.

In this case the study was carried out considering the time points of one week, one month and two months. Five units for each time point were analysed once.

LC/MS analyses

For the analyses of the organic micropollutants, an *Acquity*® ultra high pressure liquid chromatography (UHPLC) system (*Waters Corporation*, Milford, MA, USA) coupled to a hybrid triple-quadrupole linear ion trap mass spectrometer (5500 *QTRAP*®) with a turbo ion spray source from *AB SCIEX* (Foster City, CA, USA) were used. The *QTRAP*® system was operated for quantification of the target analytes in selected reaction monitoring (SRM) acquisition mode (MS/MS) with both positive and negative electrospray ionisation. Unequivocal identification was provided by the acquisition of two SRM transitions per compound in most cases (Table 4). The protonated or deprotonated molecular ion of each compound was chosen as the precursor ion.

Ultra high pressure LC separations were performed with a reversed-phase BEH C18 analytical column (*Waters*; 50 × 2.1 mm, 1.7 µm). For the analyses performed in positive mode, the compounds were separated using Milli-Q water/methanol 95:5% (v/v), with 0.1% acetic acid employed as mobile phase A and acetonitrile with 0.1% formic acid as

mobile phase B at a flow rate of 0.4 mL/min. The gradient elution started with 90% mobile phase A held for 1 min and then ramped within 8 min to 95% mobile phase B, held for 0.1 min, and then reverted to initial conditions via a 0.1 min ramp, allowing 2 min of stabilisation time. The total analysis time was 12 min.

For the analyses performed in negative mode, the compounds were separated using Milli-Q water with 0.03% ammonium hydroxide employed as mobile phase A and methanol as mobile phase B at a flow rate of 0.3 mL/min. The gradient elution started with 65% mobile phase A held for 1 min and ramped within 5 min to 50% mobile phase B, held for 2 min, then ramped up to 90% mobile phase B within 2 min and reverted to initial conditions allowing 2 min of stabilisation time. The total analysis time was 12 min.

The operating conditions for the analyses performed in both positive and negative ionisation modes were as follows: ion spray voltage 4,500 V; curtain gas 25 (arbitrary units); ion source gases GS1 and GS2 were 55 and 45 psi, respectively; probe temperature 550°C. Nitrogen served as nebuliser and collision gas.

Careful optimisation of the compound-dependent MS parameters was performed for each chemical substance. Optimisation of MS parameters (declustering potential (DP) and collision energy (CE)) was performed by flow injection analysis for each compound. The entrance potential (EP) for precursor ions and the collision cell exit potential (CXP) for product ions were not changed for any of the compounds because they had very little influence on the optimisation process. They were set to default values of 10 (EP) and 11 or 13 (CXP). The declustering potential was optimised for each compound in order to obtain the maximum response for the protonated $[M+H]^+$ or deprotonated $[M-H]^-$ molecular ion and to prevent in-source fragmentation or adducts. Table summarises the precursor ions and suitable MS/MS transitions selected by the optimisation procedure. All data were acquired and processed using the *Analyst*® 1.6 software package.

Table 4. SRM operative parameters. Q1: parent ion (m/z). Q3: product ion (m/z). ID: analyte name. DP: declustering potential. EP: entrance potential. CE: collision energy. CXP: cell exit potential.

Q1	Q3	ID	DP	EP	CE	CXP
294	250	Diclofenac	-42	-10	-16	-11
294	214	Diclofenac	-42	-10	-29	-11
300	256	13C6-Diclofenac	-173	-10	-15	-11
227	212	Bisphenol A	-120	-10	-25	-11
227	133	Bisphenol A	-120	-10	-36	-11
239	224	13C12-Bisphenol A	-120	-10	-29	-11
271	143	Estradiol	-75	-10	-66	-11
271	145	Estradiol	-75	-10	-51	-11
273	147	13C2-Estradiol	-215	-10	-54	-11
287	35	Triclosan	-69	-10	-45	-11
287	142	Triclosan	-69	-10	-50	-11

299	35	13C12-Triclosan	-69	-10	-44	-11
237	194	Carbamazepine	250	10	28	13
237	165	Carbamazepine	250	10	60	13
247	204	Carbamazepine-d10	234	10	31	13
192	91	DEET	244	10	41	13
192	119	DEET	244	10	24	13
198	91	DEET-d6	80	10	42	13
233	72	Diuron	169	10	25	13
233	133	Diuron	169	10	53	13
240	78	Diuron-d6	156	10	24	13
254	156	Sulfamethoxazole	150	10	22	13
254	92	Sulfamethoxazole	150	10	38	13
260	98	13C6-Sulfamethoxazole	70	10	36	13

GC/MS analyses

Atrazine, chlorfenvinphos, chlorpyriphos, DEHP, fluoranthene, isoproturon, 4-nonylphenol and simazine analyses were carried out with a gas-chromatograph coupled with a single quadrupole mass spectrometer, (GC-MS, Agilent model 6890 coupled to an MSD 5975 inert mass-selective detector) operating with EI ionisation in the selected ion monitoring (SIM) mode. The column used was a DB 5MS, 30 m x 0.25 mm ID and 0.25 µm film thickness. The carrier gas was helium at a constant flow rate of 1.2 mL min-1. The injector was operated in splitless mode at a temperature of 270°C. The GC oven was programmed as follows: 60°C hold for 1 min; increase at a rate of 30°C min-1 to 130°C; hold at 130°C for 1 min; increase at 7°C min^{-1} to 180°C; hold at 180°C for 6 min, increase at a rate of 15°C min^{-1} to 280°C and hold at 280°C for 0 min, increase at a rate of 20°C min^{-1} to 310°C and hold at 310°C for 8 min. The interface temperature was maintained at 280°C.

ICP-MS analyses

Ni and Cd were measured using a quadrupole ICP-MS Agilent 7500ce, equipped with octopole collision/reaction cell in order to minimise the isobaric and spectral interferences. The matrix effect was corrected using the internal standard technique. The instrumental conditions of the ICP-MS were according to the manufacturer's instructions. External calibration and quality control were applied using reference solutions certified in according to ISO Guide 34 and ISO 17025.

Data evaluation

The results for all the candidate reference materials were plotted against time. A screening of any suspicious data was performed and the regression line for the substance stability

versus time was calculated. Both slope and regression lines were tested for statistically significant trends.

3.2 Reconstitution protocol

A kit containing the five reference materials has been distributed to all partner laboratories, each in vials containing 2 mL of reference material. The number of kits received by each laboratory was according to the needs of the specific bioassays to be performed.

The reference materials were dispatched in dry ice by express courier, and in all cases, the vials were still in dry ice upon arrival. The reference organic mixtures were kept at the reference temperature (-20°C) until reconstitution, and inorganic solutions were kept at 4°C. A reconstitution protocol has been distributed to all laboratories.

The reconstitution of the reference materials (ISPRA RM040-044) into final Exposure Mixtures Mix14 and Mix19 is described in Table 5. An indicative dilution of 1:1000 and a final volume of the Exposure Mixtures of 1 litre are shown. The final solvent of the mixtures was either Milli-Q water or the media specifically required for each bioassay. In some cases it was required to adjust the pH of the mixtures and the solvent control (SC) prior to the initiation of the bioassay, to avoid a toxic response from the methanol/nitric acid solvent to the organisms. After reconstitution, Mix14 and Mix19 were kept at 4ºC until use. Some laboratories chose to extract the chemicals after reconstitution in Milli-Q water to mimic the normal procedures of analysis of environmental water samples, as indicated. For several bioassays, a dose-response has been performed for each of the mixtures, with several concentrations tested. In this case, the final concentrations are expressed as the fold concentration relative to 1×EQS.

Table 5. Reconstitution of the concentrated reference materials (ISPRA RM) into 1 L of the final mixtures or solvent control. Each final mixture was produced by adding one organic and one inorganic 1000-concentrated reference mixtures to the appropriate assay buffer.

	Mix14 (10xEQS)	Mix14 (1xEQS)	Mix19 (1xEQS)	Solvent Control
ISPRA RM040	1 mL	-	-	-
ISPRA RM041	-	1 mL	-	-
ISPRA RM042	-	-	1 mL	-
ISPRA RM043	1 mL	-	-	-
ISPRA RM044	-	1 mL	1 mL	-
Methanol	-	-	-	1 mL
2% nitric acid	-	-	-	1 mL
Milli-Q water or buffer	1L	1L	1L	1L

4. BIOASSAYS

4.1 Community-based assay – marine microcosm

The community-based assay tested the time-dependent changes in the composition of the marine microcosm in the presence of the chemical mixtures.

Sea water was collected in the middle of the Gulf of Trieste (45° 32' 55, 68'' N, 13° 33' 1, 89'' E; northern Adriatic Sea, North-East Mediterranean) at depth of chlorophyll maximum (20 m) on 18th July 2013. Sampling was performed by Niskin sampler and the seawater was pre-filtered through 53 µm acid-washed Nitex to remove larger phytoplankton grazers. The time zero (T0) sample corresponded to the sea water as collected in the environment. All samples were kept protected from light at environmental temperature (19°C) and were brought to the Marine Biology Station within 1h.

Sea water samples were then distributed into experimental bottles (1 L acid washed bottles), to which the reference mixtures were added to produce Mix14 (at 1X and 10X EQS) and Mix19 (at 1X EQS), each treatment in triplicate. At the same time two sets of controls were prepared: untreated seawater (SW) and solvent control (SC, methanol and 2% nitric acid added to 1 L seawater). All bottles were incubated in a thermostatic room at constant temperature (15°C) and day/night light conditions. pH was corrected to reach the standard seawater pH 8.3 with 0.1M NaOH.

Samples were taken after 6, 12, 24 and 48 hours, in equal volumes from each of the triplicate bottles and analysed for bacterial abundance, bacterial production and phytoplankton pigment analyses.

Bacterial carbon production (BCP) was measured using ^{3}H-leucine incorporation method (Smith and Azam, 1992) and expressed as the number of cells/L/h, using 20 fg C bacterium^{-1} as conversion factor.

The qualitative and quantitative analyses of pigments in the water samples were determined using a reverse-phase HPLC (High Performance Liquid Chromatography) method (Mantoura and Llewellyn, 1983; Barlow et al. 1993). Water samples were filtered through Whatman GF/F filters and immediately frozen at -80 °C until analysed. Frozen samples were extracted in 90% acetone, sonicated and centrifuged 10 min at 4000 rpm to remove particles. The supernatant was mixed with 1 M ammonium acetate (1:1), the pigments were separated by RP-HPLC using a 3 µm C_{18} column (Pecosphere, 35x4.5 mm, Perkin Elmer) and detected by absorbance at 440 nm using a diode array detector. The data were statistically evaluated using two-way ANOVA.

4.2 Organism toxicity assays

4.2.1 *Vibrio fischeri*

The assay measures the inhibition of bioluminescence emitted naturally by the bacterium *Vibrio fischeri* exposed to aqueous samples and is based on the EN ISO 11348-3 guideline.

Luminescence inhibition in % was calculated as follows:

$$Ht = \frac{Ict - ITt}{Ict} * 100$$

where *Ht* is the inhibitory effect of sample after contact time, *ITt* is luminescence intensity of sample after contact time, *Ict* is corrected value *I*0 of control sample before incubation time:

$$Ict = I0 * Fkt$$

where $I0$ is luminescence intensity of control suspension immediately before addition of diluent, *Fkt* means correction factor (natural extinction of luminescence) calculated as follows:

$$Fkt = \frac{Ikt}{I0}$$

where *Ikt* is luminescence intensity of control sample after the contact time. $I0$ is luminescence intensity of control sample immediately before the addition of diluent

RECETOX

Samples were prepared and mixed according to the reconstitution protocol in glass volumetric flasks. Each sample was adjusted to achieve neutral pH in the range 6.5-7.5. Samples were tested in 75% of original concentration, the dilution was due to addition of sodium chloride solution to establish salty environment. Solution of NaCl (2%) was used as negative control. 30 µL of bacterial suspension (*Vibrio fischeri* NRRL-B-11177, LUMIStox luminous bacteria, Germany) was pipetted into 96-well white microplate (polystyrene – plastic). Initial luminescence of bacteria was recorded. Subsequently, 120 µL of samples, negative and positive controls were added into each well. Luminescence was recorded after 15 and 30 minutes of exposure. The whole test was carried out at 15°C. Biotek Synergy™ microplate luminometer was used for luminescence measuring.

Three independent runs of the test were performed. Mean, standard deviation and number of replicates were used for statistical evaluation using GraphPad QuickCalc on-line software, statistical significance of differences between control and exposure mixtures was tested by unpaired *t*-test.

ECOTOX

In the bacterial bioluminescence inhibition test the bacteria *Vibrio fischeri* were exposed to samples in 96-well plates. The endpoint recorded in a microplate luminometer after 30 min of exposure represents a non-specific toxicity and indicates an intervention in the energy metabolism of the bacteria. 3,5-dichlorophenol was used as positive control and the data are expressed as EC_{10} and EC_{50} (the concentration causing 10% and 50% of the maximum effect, respectively).

The evaluation of the generated data by fitting a dose response curve was carried out with GraphPad Prism 5 Software (La Jolla, CA, USA). This fit provided EC_{50} and EC_{10} values.

The pollutants reference materials were reconstituted in nanopure water following the common reconstitution protocol and treated according to the usual laboratory procedure. The samples were enriched by performing a SPE (solid phase extraction) and eluted with acetone and methanol. For the storage of the samples (-20°C) the acetone/methanol mixture was evaporated and the samples were redissolved in 1 mL ethanol. Depending on a range finder performed in advance, 8 dilutions of the extracted samples were tested in triplicates within the next three weeks.

ISPRA

The tests were conducted with the luminescence bacteria *Vibrio fischeri* NRRLB-11177. Freeze-dried bacteria were provided by Ecotox Ltd. (batch n° 12J4123, expiry date 10/2014).

The Microtox® system was used to perform the test. This combines, into cuvettes, specific volumes of the test sample (or diluted sample) with bacterial suspension at initial concentration of 10^6 cells/mL. The exposure mixtures (Mix14 at 1X EQS and 10X EQS, and

Mix19 at 1X EQS) and the solvent were prepared according to the reconstitution protocol received using a solution obtained by mixing 10 mL of osmotic solution (NaCl 22%) and 80 mL of deionised water (conductivity <10 μS/cm). The pH of test samples and the solvent was not considered compatible with the survival of luminescence bacteria, making necessary an adjustment with 0.1-1 N NaOH to values ranging 6.1-6.9.

The luminescence of bacteria was measured after contact time of 5, 15 and 30 minutes. The results are provided as percentage difference between mean values obtained in the control and in the test samples.

Potassium dichromate ($K_2Cr_2O_7$) was used as positive control with an EC_{50}-30min value of 30.7 mg/L (95% confidence interval: 26.7-35.4 mg/L) that was within the range recommended by the ISO 11348-3.

UNIPMN

The tests with the luminescent bacterium *Vibrio fischeri* were performed according to the method IRSA - CNR, ISSN:0392-1425 (1996), using 1 mL of each mixture reconstituted according to the common protocol. The reduction of light emission of bacteria after exposure for 15 minutes to the mixtures (Mix14 at 1X EQS and 10X EQS, and Mix19 at 1X EQS) was measured with a Microtox® luminometer.

4.2.2 *Pseudokirchneriella subcapitata*

The algal growth inhibition test using the green alga *Pseudokirchneriella subcapitata* followed the standard ISO 8692 in 96-well microplates.

RECETOX

A modified standard EN ISO 8692:1989 was used with the 50% ZBB medium (prepared by mixing Zehnder Z-medium, Bristol's modified Bold's medium and distilled water in the ratio of 1:1:2). The suspension of the cells in exponential growth phase was mixed 1:1 with the exposure mixture (two-fold concentrated) and pipetted into the plate, ten (10) replicates per mixture (Mix14 at 1X and 10X EQS, and Mix19 at 1X EQS), untreated control and solvent control. The plate was incubated at 23°C under continuous light. Absorbance at 680 nm was measured at the beginning and the end of the test (96 hours) when the average growth was calculated and the growth inhibition in percentage of control was evaluated.

ISPRA

The growth inhibition test was performed according to the ISO 8692:2012 with the unicellular green alga *Pseudokirchneriella subcapitata* (purchased as strain 61.81 at the Culture Collection of Algae in Gottingen).

The exposure mixtures (Mix14 at 1X and 10X EQS, and Mix19 at 1X EQS) and the solvent were prepared according to the reconstitution protocol, after adjusting the pH with 0.1-1 N NaOH, to values ranging between 7.8 and 8.3.

The algal assay was conducted using six multi-well plates for cell culture (volume: 10 mL/well). Six and three replicates for control and treated samples, respectively, were prepared. All plates were incubated in a temperature-controlled cabinet (23±2°C) under continuous white light (8000 lux light intensity) and under shaker conditions to facilitate CO_2 mass transfer from air to water.

The initial cell density was about 10^4 cells/mL of *P. subcapitata* in exponential growth phase. Algal density (cells/mL) was measured in each well after 24, 48 and 72 hours of incubation by the Coulter Counter Z1 Laboratories Instruments (thresholds: 2.5-9.5 μm^3 spherical diameter).

Growth inhibition in the test samples was measured as reduction in specific growth rate relative to control cultures after 72 hours of exposure.

In the control replicates, the average growth rates were in the range 1.9–2.2 d^{-1}, the variation coefficient of the growth rate was in the range 0.1-0.3% and pH variation did not exceed 0.4 units.

DTU

P. subcapitata assays were conducted using a modified version of the ISO 8692:2012 with alga obtained from the Norwegian Institute of Water Research, Oslo, Norway. The tests were performed in a mini-scale version of the ISO test with 4 mL test solution in 20 mL test glass vials (acid washed) capped with lids with a small hole allowing equilibration with atmospheric CO_2 while minimising volatilisation losses from solution. The tests were conducted with 5 concentrations for each mixture in 3 replicates and control groups in 6 replicates. The initial cell density was 10^4 cells/mL of *P. subcapitata* in exponential growth phase. Vials were incubated on a shaker (app. 200 rpm) in continuous light (83-108 $\mu E/m^2/s$) at 21±2°C. Biomass was determined in each vial after 0, 24, 48, and 72 hours incubation by algal pigment fluorescence measurements with a Hitachi F-2000 Fluorescence Spectrophotometer (Hitachi, Japan) at 420 nm excitation light and 671 nm emission light after pigments extraction with acetone as described by Mayer et al. (1997). The average growth rates of the control groups were in the range 1.21-1.32 d^{-1} and pH variation did not exceed 0.5 units. A reference test performed with potassium dichromate ($K_2Cr_2O_7$) provided an EC_{50} value of 0.85 mg/L $[0.76; 0.93]_{95}$%, which was within the usual range of the laboratory. In the algal test, curves were described by the log-normal model, which was fitted to data using nonlinear regression by applying a computer program based on the principles described by Andersen et al. (1998).

ECOTOX

In a combined algae test, *P. subcapitata* were exposed to samples in 96-well plates and two endpoints were reported. The main endpoint was recorded in the maxi imaging PAM (IPAM, Heinz Walz GmbH, Germany) after 2 hours of exposure and represents the inhibition of photoysystem II (PS II). Diuron was used as a reference compound and the data were expressed as EC_{10} and EC_{50} (the concentration causing 10% or 50% of the maximum effect) as well as DEQ (diuron equivalent concentration). With this endpoint one captures the combined effects of compounds such as diuron, atrazine, simazine, etc. The second endpoint reflects toxic effects on the algal growth rate. The inhibition of the growth rate was determined by measuring the OD_{685} after an exposure of 0 h, 24 h and two additional intermediate reading points. The growth rate inhibition endpoint was expressed as EC_{10} and EC_{50}.

The evaluation of the generated data by fitting a dose response curve was carried out with GraphPad Prism 5 Software (La Jolla, CA, USA). The fit provided the EC_{50} value and out of this the EC_{10} and DEQ values were calculated.

UNIPMN

The green algae bioassay was performed according to the method ISO 8692:2005. Algal cultures of *P. subcapitata* in exponential growth phase (10^6 cells/mL) were incubated in a volume of 2 mL of each exposure mixture (Mix14 at 1X and 10X EQS, and Mix19 at 1X

EQS). Growth inhibition was measured by cell counting after exposure for 72 h to the different samples.

All tests were performed in four replicates and significant differences were tested using the non-parametric Mann–Whitney *U* test.

4.2.3 *Chlamydomonas reinhardtii*

EAWAG

For the toxicity assays, *C. reinhardtii* in the log phase of growth at a cell density of $2.5x10^5$ cells/mL in growth medium were exposed to Mix14 to estimate the dose response curve. All exposures were done in a volume of 20 mL in Erlenmeyer flasks to obtain enough algal material for RNA isolation to estimate molecular endpoints. The mixture Mix14 was prepared by direct dilution of ISPRA RM040/043 in the medium to varying concentrations ranging from 0.2x to 100x EQS. The control algae were exposed to methanol at different concentrations. After exposure for 24 h, physiological responses such as the growth rate and photosynthetic yield were estimated.

4.2.4 *Thalassiosira pseudonana*

JRC

T. pseudonana (strain CCMP 1335) was obtained as axenic culture from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP, West Boothbay Harbour, Maine, USA). Diatoms were maintained at around 14°C under a diurnal light cycle of 13 h light and 11 h darkness. The culture medium was f/2 medium, based on artificial sea water with a salt concentration of 32 g/L sodium chloride (NaCl) (ASW, Sigma-Aldrich, Steinheim, Germany). *T. pseudonana* cultures were first synchronised according to Hildebrand et al. (2007). Cultures with a cell density of 10^6 cells/mL were exposed to a dose-response of Mix14, with final concentrations ranging between 1x and 20x EQS, in triplicates. Cell densities were determined after 24, 48 and 72 h by measuring the absorption at 450 nm using a microplate spectrophotometer (Biorad, Hercules, CA, USA). Diatom growth rates were calculated as previously described (Bopp and Lettieri, 2007) and the inhibitory effect of the mixtures or solvent on the growth rate of diatom cultures was calculated by comparing to the untreated cultures.

4.2.5 *Daphnia magna*

4.2.5.1 Acute immobilisation test (ISO 6341)

Newly hatched neonates (less than 24 hours old) obtained from the continuous laboratory culture were used (20 animals were used per exposure condition (Mix14 at 1X and 10X EQS, and Mix19 at 1X EQS, untreated control and solvent control), divided into four replicates, each containing five organisms.

RECETOX

Acute immobilisation test with *D. magna* was based on ISO 6341:1996. For the breed (as well for the test), the ADaM medium (Aachener Daphnien Medium) was used, imitating natural fresh water (Klüttgen et al. 1994) in glass beakers. The total duration of the exposure was 48 h, and immobilised organisms were counted after 24 and 48 h. The number of immobilised animals after 48 hours was taken as the final reported endpoint value. Results were expressed as the percentage of the untreated control. Mean, standard deviation and number of replicates were used for statistical evaluation using GraphPad

QuickCalc on-line software, statistical significance of differences between control and exposure mixtures was tested by unpaired *t*-test. Two independent runs of experiment were performed with the same results. With respect to positive result obtained for Mix14 at 10X EQS, additional dilutions were tested (100, 50, 25, 12.5 and 6.25% v/v).

ISPRA

Acute toxicity tests with the crustacean *Daphnia magna* Straus were performed according to the ISO 6341:2012. Test organisms were purchased as dormant eggs (ephippia) immersed in a preservation medium (Daphtoxkit MicroBioTest Inc., Ecotox srl). Neonates less than 24 hours old were derived in our laboratory from the hatching of ephippia three days before the start of the exposure.

The exposure mixtures (Mix14 at 1X and 10X EQS, and Mix19 at 1X EQS) and the solvent were prepared according to the reconstitution protocol. The pH was adjusted with 0.1-1 N NaOH to values ranging between 7.0 and 8.1.

Multi-well test plates in polycarbonate with 10 mL of medium were used for the exposure. After 24 hours (20°C in darkness), the number of immobilised animals was counted and the effects were expressed as percentage of immobile organisms.

A reference test carried out with potassium dichromate ($K_2Cr_2O_7$) provided an EC_{50} 24 h value of 1.42 mg/L (95% confidence interval: 1.06-1.90 mg/L) that was within the range recommended by the standard.

DTU

Acute toxicity tests on the freshwater crustacean *D. magna* were carried out according to the ISO 6341:2012 except for the test media, which were made according to the OECD TG 211 (2012). Glass beakers (100 mL) with five neonates (age <24 h) were used per exposure condition. Glass beakers were covered with glass lids and kept in the dark at 18-22°C. The number of immobile animals was counted after 24 and 48 h. In tests with the reference compound potassium dichromate ($K_2Cr_2O_7$), the 24 h EC_{50} was 1.8 mg/L (95% CI, 1.7–1.9 mg/L), fulfilling the validity criteria in the ISO standard of an EC_{50} between 0.9 to 2.4 mg/L. For calculation of the concentration-response relationships in tests with *D. magna*, the ToxCalc™ software v5.0 (Tidepool) with maximum likelihood logit regression was used.

4.2.5.2 Reproduction test with Daphnia magna

RECETOX

The reproduction test with *D. magna* was conducted according to the standards OECD TG 211 (1998) and the CSN ISO 10706 (2001) with modifications. As in the acute test, the ADaM medium (Klüttgen et al. 1994) was used. Newly hatched daphnids from the continuous laboratory culture were transferred into the glass beakers (10 animals for each exposure mixture, control and solvent control, one animal separately per beaker) with 50 mL of the exposure mixture. During 21 days of exposure, the survival and the reproduction were monitored. Exposure mixtures were changed three times a week and daphnids were fed with the mixture of green algae (*Pseudokirchneriella, Chlorella* and *Scenedesmus* spp.). Experiments were run at temperatures 21±1°C and photoperiod 16:8 light/dark. Offspring produced by parent animals were counted and removed. Survival of parent animals and number of live offspring were evaluated and expressed as a percentage of control. Mean, standard deviation and number of replicates were used for statistical evaluation using GraphPad QuickCalc on-line software, statistical significance of differences between control and exposure mixtures was tested by unpaired *t*-test.

4.2.6 *Dictyostelium discoideum*

UNIPMN

The amoebic cells of *D. discoideum* (0.75 x10^6 cells/m) were incubated with 3 mL of each mixture (Mix14 at 1xEQS and 10xEQS, and Mix19 at 1xEQS) and 1 mL of AX-2 culture medium. Cell viability and lysosomal membrane stability (LMS) were evaluated after 3 h of exposure while the replication rate was assessed after 24 h of treatment. Cell viability and replication rate were carried out as described by Dondero et al. (2006), LMS as described by Sforzini et al. (2011).

4.2.7 *Danio rerio* - Fish Embryo Toxicity test (FET)

RECETOX

The test is based on EN ISO 15088 guideline and OECD TG236 guideline for Fish Embryo Toxicity (FET) test with some modifications. Zebrafish (*Danio rerio*) embryos were used for the experiments. Adults were kept in conditions according to the ISO 15088. Embryos were collected just prior to the start of the experiment and immediately exposed. Embryos were exposed to Mix14 at 1X and 10X EQS, and Mix19 at 1X EQS, under static conditions for five days, ten embryos per 40 mL media (standard ISO medium was used), three replicates per exposure mixture plus control and solvent control. Two independent runs of experiment were performed starting on different days. Embryos were observed daily. Endpoints specifically observed after 120 hours (total mortality, number of hatched, number of defected, head deformations, tail deformations, absence of gas bladder, underdeveloped, and length) were reported. Original data were used for statistical evaluation that used ANOVA followed by Dunnett and Fisher LSD post hoc test (for data in individual experimental runs). Homogeneity and normality was tested by Levene and Shapiro-Wilk tests. Nonparametric Kruskal-Wallis test was used for data without normal distribution and Chi-square test was used for testing differences in frequencies. STATISTICA for Windows (StatSoft) and Microsoft Excel were used for calculations.

4.2.8 Xenopus laevis - Frog Embryo Teratogenesis Assay (FETAX)

RECETOX

The test was based on ASTM E1439-98 guideline with modifications (Jin et al. 2010). *Xenopus laevis* adults used in this test were born in RECETOX's laboratories and maintained in plastic tanks in dechlorinated tap water. Males and females were placed in tanks in mixed groups (males and females together), four animals per tank. They were fed with mixture of ground beef liver, lung and heart with gelatine and reptile multivitamin mix. Room and water temperature was 19°C, 12 hours day/night rhythm.

Two pairs were placed for breeding in separated plastic breeding tanks with standard FETAX medium. The tanks were equipped with plastic nets, thermostats set to 23°C and bubblers. Both males and females were stimulated with HCG (human chorionic gonadotropin – females 500 IU and males 300 IU, respectively) in the form of Pregnyl 5000 medicament (N.V. Organon, The Netherlands) injected into dorsal lymph sac. Eggs were staged according to Nieuwkoop and Faber (1994). After reaching the stage 46, they were manually collected from the tank with plastic dropper (Pasteur pipette). Normally cleaving embryos were selected for the test. No chemical dejellying was applied.

The test was performed in safety hood under constant temperature (20°C) and low light. Embryos were manipulated with plastic droppers. Sterile plastic Petri dishes were used for the exposure. Each dish contained 30 embryos and 10 mL of solution, five replicates for each exposure mixture and solvent control. Solutions were changed every 24 hours, and dead embryos removed periodically. After 96 hours, embryos from each dish were moved

to test tubes and anesthetised with 5 mL of 100 mg/L tricaine methanesulfonate. After anesthesia (no movement) they were fixed with 5 mL of 3% formaldehyde.

Four parameters were evaluated: mortality, embryo length, number and type of malformations. Total mortality was compared to the original number of eggs that survived after 48 hours (this is because some eggs were unfertilised or severely malformed although they appeared normal at stage 46). For the diagnosis of malformations, Atlas of Abnormalities (Bantle, 1991) was used as a reference guide. The embryos were observed with light microscope, digitally photographed and measured with QuickPhoto MICRO software (Promicra, Prague, Czech Republic).

4.3 *In vitro* toxicity assays

4.3.1 Tetrazolium MTT test

The MTT assay is a basal cytotoxicity assay that assesses the ability of cells to reduce the yellow tetrazolium salt (MTT) to formazan. This ability indicates an integrity and functionality of the cellular mitochondria.

4.3.1.1 MTT test with human cells

BOKU

Cell lines

The use of human cell lines to assess human toxicity is of high interest. Human primary cells offer a high similarity to the *in vivo* situation, however, their growth is limited by the phenomenon of replicative senescence to a certain amount of population doublings (Hayflick and Moorhead, 1961). Therefore, cells have to be continuously isolated from donors, with sometimes difficult supply of tissues and high variability of assays due to variations in the donor backgrounds.

Several immortalised cell lines have been established recently that maintain all key functionalities of the human primary cells, but still show continuous growth and thus unlimited cell supply. This is achieved through counteracting replicative senescence by over-expressing the catalytic subunit of human telomerase and stabilising/elongating telomeres in the cells (hTERT) (Bodnar, 1998). A large variety of different human cell lines such as fibrobralsts (Bodnar, 1998), retinal epithelial cells (Bodnar, 1998), endothelial cells (Chang et al. 2005) or renal proximal tubular epithelial cells (Wieser et al. 2008) have already been established by introduction of hTERT.

RPTEC/TERT1: human renal proximal tubular epithelial cells

The kidney is one of the main targets of organism toxicity along with the liver and the heart. RPTEC/TERT1 cells have been recently immortalised and characterised on a functional level showing high similarity to the normal, mortal counterparts (Wieser et al. 2008), thus they have been used in several studies for assessing kidney toxicity (Ellis et al. 2011; Jennings et al. 2012; Limonciel et al. 2011 and 2012; Radford et al. 2012; Wilmes et al. 2013) in the context of pharmaceutical research.

RPTEC/TERT1 cells (Evercyte GmbH) were cultivated in ProxUp medium (Evercyte GmbH). For passaging, the medium was removed and the cell layer was washed with phosphate buffered saline (PBS, PAA) twice. Then, 0.5 mL of 0.25% trypsin (Invitrogen) + 0.02% EDTA (Sigma-Aldrich) were added per T25 culture flask and cells were incubated at 37°C until detachment (approximately 5 min). After complete detachment, 0.25 mL trypsin inhibitor (Sigma-Aldrich) was added and cells were resuspended in 2-3 mL of culture medium and centrifuged for 5 min at 170 *g*. The supernatant was discarded, the cell pellet

was resuspended in fresh medium and seeded into new culture flasks according to the cell line and population doubling level. A split ratio of 1:3 was applied twice a week.

HUVEC/TERT7: human umbilical vein endothelial cells

Endothelial cells are important to assess toxicity, as substances that have entered the circulation come into contact with this cell type. Human umbilical vein endothelial cell (HUVEC) lines have been established by introducing hTERT, and were shown to maintain high population doubling levels similar to the normal counterpart cells (Chang et al. 2005). This cell line so far has not been used in toxicity studies, although HUVECs are already in use in various fields of toxicology, including drinking water safety assessment (Gutiérrez-Praena et al. 2012), ecotoxicology in regard to asbestos (Carbonari et al. 2011), carbon nanotubes (Gutiérrez-Praena et al. 2011), polychlorinated biphenyls (Andersson et al. 2011) or Diesel exhaust (Chao et al. 2011).

HUVEC/TERT7 cells (Evercyte GmbH) were cultivated in EndoUp-2 medium (Evercyte GmbH) in culture dishes pre-coated with gelatine (1% in PBS). For passaging, the medium was removed and the cell layer was washed twice with PBS. Then, 0.5 mL of 0.1% trypsin (Invitrogen) + 0.02% EDTA were added per T25 culture flask and cells were incubated at 37°C. After complete detachment (approximately 5 min) cells were resuspended in fresh culture medium and seeded into new culture flasks. A split ratio of 1:4 was applied twice a week.

HepG2: hepatocellular carcinoma cells

HepG2 cells (ATCC) were cultivated in DMEM/Ham´s F12 (Biochrom) supplemented with 4 mM L-glutamin (Biochrom) and 10% FCS (PAA). For passaging, the medium was removed and the cell layer was washed with PBS twice. Then, 0.5 mL of 0.25% trypsin (Invitrogen) + 0.02% EDTA were added per T25 culture flask and cells were incubated at 37°C. After complete detachment (approximately 5 min) cells were resuspended in fresh culture medium and seeded into new culture flasks. A split ratio of 1:3 was applied twice a week.

MCF-7: breast cancer cell line

MCF7 cells (ATCC) were cultivated in MEM Earls Salt (Biochrom) supplemented with 2 mM L-glutamin (Biochrom), 1 mM Na-pyruvate (Biochrom), 1x non-essential aminoacids (Biochrom), 0,01 mg/mL insulin (Sigma-Aldrich) and 10% FCS (PAA). For passaging, the medium was removed and the cell layer was washed with PBS twice. Then, 0.5 mL of 0.25% trypsin (Invitrogen) + 0.02% EDTA were added per T25 culture flask and cells were incubated at 37°C. After complete detachment (approximately 5 min) cells were resuspended in fresh culture medium and seeded into new culture flasks. A split ratio of 1:3 was applied twice a week.

Exposure

Cells were seeded in the inner wells of a 96-well plate. The outer wells were filled with 200 µL PBS to avoid evaporation of the medium. When the cells had reached about 70-90% confluency, treatment medium was applied. Mix14 was tested at concentrations of 1×EQS and 10×EQS, while Mix19 was tested at 1×EQS. Incubation time was set to 24 h at 37°C and 5% CO_2. Thereafter, 10 µL/well MTT-solution (2 µg/µL, Promega) were added and cells were incubated for another 4 h. 100 µL of 10% SDS in 0.01 M HCl were added to the wells. The plates were incubated for another 20 h; then the absorption was measured at 570 and 690 nm with an Infinite® M200 microplate reader (Tecan, Männedorf,

Switzerland). The value for the reference wavelength of 690 nm was then subtracted from the absorption at 570 nm.

4.3.1.2 MTT test with RTG-2 rainbow trout gonad cells

ISPRA

The method used for the assay (Figure 2) was developed on the basis of the Protocol No. 17 INVITTOX ECVAM and scientific literature (Caminada et al. 2006; Davoren et al. 2007; Jin et al. 2010; Twentyman and Luscombe, 1987).

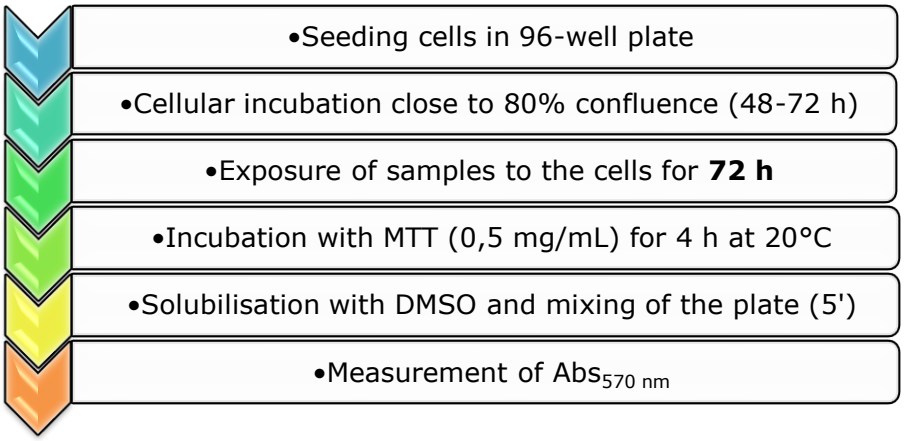


Figure 2. MTT assay with RTG-2 rainbow trout gonad cell.

The test samples (Mix14 at 1X and 10XEQS, and Mix19 at 1XEQS) and the solvent control (SC) were reconstituted in the culture medium of the cells. The pH of the mixtures and that of the solvent was not compatible with cell survival and was therefore adjusted to pH 7.2-7.6.

The measurements of absorbance (Abs) were performed with the spectrophotometer TECAN Infinite F200Pro at the wavelength of 570±10 nm (shaking duration: 5, multiple reads per well: circle filled 5x5, number of flashes per well: 3) and corrected with respect to white.

The toxicity was expressed as the percentage of cell survival after 72 h exposure. Cell survival was determined by the ratio: [(Abst / ABSC) x100]. Abst = average value of Abs in the treatments; ABSC = average value of Abs in the controls.

The compliance of the procedure was evaluated by performing a test with a reference toxicant cadmium chloride monohydrate ($CdCl_2$). Table 6 shows the values of internal reference for this toxic and interlaboratory variability (n = 6).

Table 6. Internal reference values for $CdCl_2$ in the MTT test with RTG-2 cells.

		Mean (n= 6)	ST	CV%
EC_{50} (72 h)	mg/L	15.96	4.40	6.29
lim inf 95%	mg/L	6.23		
lim sup 95%	mg/L	25.64		

4.3.1.3 MTT test with primary rainbow trout gill cells

DNSC

Fish Gill Cell Culture System (FIGCS)

Gill cells for primary cultures were derived from juvenile rainbow trout (*Oncorhynchus mykiss*; 80-100 g) obtained from Padworth Trout farm, Berkshire, UK. All fish were housed at King's College London where they were maintained in fibreglass tanks (1000 L) with flowing and aerated de-chlorinated City of London tap water ($[Na^{4+}]$=0.53 mM; $[Ca^{2+}]$=0.92 mM; $[Mg^{2+}]$= 0.14 mM; $[K^{+}]$= 0.066 mM; $[NH_4^{+}]$= 0.027 mM) which was passed through activated carbon, mechanical and biological filters. Water temperature was maintained at 14-16°C, while photoperiod was held constant (16/8 h light-dark). Fish were fed daily a one-percent (w/w) ration of trout pellets.

The primary gill cells were isolated and cultured as described in Fletcher et al. (2000) and prepared using the double seeding technique as described in Kelly et al. (2000) and Walker et al. (2007). Isolated gill cells were seeded onto permeable transwell inserts at a cell density of 1.2 x 10^6 per cm^2 and the transepithelial electrical resistance (TEER) was measured daily in order to monitor the development of the epithelium using a voltohmmeter (EVOMX; World Precision Instruments, Sarasota, USA) fitted with STX-2 electrodes. A TEER >5 kΩ was used as criterion for the presence of a tight epithelium (Fletcher et al. 2000). Once a tight epithelium was formed, the exposure experiment was started.

Reconstitution of exposure mixture

Reaction Mix14 10XEQS and solvent control were prepared as instructed using reconstituted freshwater according to the OECD guideline n. 203 and the pH was corrected to 7.3.

Cell viability

Cell viability was assessed using the MTT assay. In healthy cells, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) is reduced by dehydrogenase enzymes forming intracellular formazan, which can be quantified spectrophotometrically. MTT (5 mg/mL) was prepared in OECD water, diluted in the ratio of 1:10 and added to the inserts for a total of 4 h. Once the incubation time was over, the solution was aspirated and the resulting formazan crystals dissolved in 500 µL of DMSO and incubated at room temperature for 30 min. The absorbance was measured at 570 nm.

4.3.2 Neutral Red (NR) test

This test compares the cytotoxicity of a chemical when tested in the presence and in the absence of exposure to non-cytotoxic UVA light. Cytotoxicity is measured as an inhibition of the capacity of the cell culture to take up a vital dye, neutral red.

RECETOX

For analysis of acute cytotoxicity, the H4IIE-luc cells were used as a model. The H4IIE-luc were plated in DMEM-F12 with phenol red (Sigma-Aldrich, USA) containing 10% fetal calf serum and the density of cells was 15000 per well. After 24 h cells were exposed to the mixtures (Mix14 at 1X and 10XEQS, and Mix19 at 1XEQS) and the solvent control (SC). At the end of the test, exposure medium was removed and 100 µL of medium with neutral red (0.05 mg/mL) was added to each well. After 1 hour of incubation, medium with neutral red was removed and lysis buffer for neutral red was added. Plates were shaken for 20 minutes and absorbance was measured at 570 nm.

4.3.3 xCELLigence systems

NIFES

Isolation of primary cultures of hepatocytes from juvenile Atlantic salmon (Salmo salar)

Juvenile Atlantic salmon (*Salmo salar*) was obtained and kept at the fish rearing facility at Matre Research Station which belongs to the Institute of Marine Research, Bergen, Norway. The fish were fed once daily with a special feed produced without addition of synthetic antioxidants and with low levels of contaminants, supplied by EWOS, Norway (Harmony Nature Transfer 75). All glassware, instruments and solutions were autoclaved prior to liver perfusion. Hepatocytes were isolated from 8 Atlantic salmons (288-375 g) with a two-step perfusion method previously described by Søfteland et al. (2009). The final cell pellet was resuspended in L-15 medium containing 10% fish serum (FS) from salmon (Nordic BioSite, Oslo, Norway), 1% glutamax (Invitrogen) and 1% penicillin-streptomycin-amphotericin (10000 units/mL potassium penicillin, 10000 µg/mL steptomycin sulfate and 25 µg/mL amphotericin B) (Lonzo, Medprobe). The Trypan Blue exclusion method, performed in accordance with the manufacturer's protocol (Lonzo), was used to determine cell viability. The different cell suspensions used in this study had cell viability between 86-92%. The cell suspensions were plated on 2 µg/cm^2 laminin (Sigma-Aldrich) coated culture plates (TPP, Trasadingen, Switzerland), and the hepatocytes were kept at 10°C in a sterile incubator without additional O_2/CO_2 (Sanyo, CFC FREE). Cell concentrations of 0.2×10^6 cells per well in xCELLigence 96-well plates (in 0.2 mL complete L-15 medium) were used.

Chemical exposure

The primary cells were cultured for 36-40 hours prior to chemical exposure with a change of medium (containing 10% FS) after 18-20 hours. The cells were exposed for 24 hours to Mix14 at 1X and 10X EQS, and Mix19 at 1X EQS, and the solvent control (SC). Cells from four fish were used per treatment. The exposure medium contained 1% FS and was substituted with new medium after 18-20 hours. The chemical exposure was sustained for another 24 hours.

Cytotoxicity assessment

For the cytotoxicity assessment of the four chemical compounds, real time impedance data obtained by the xCELLigence systems (Roche Diagnostics) was applied. The xCELLigence system quantifies electrical impedance across electrodes in 96-well cell culture E-Plates. The impedance measurement gives quantitative information regarding cells' biological status including morphology, cell number and viability. After a background reading was measured, the appropriate number of cells was added to the plate. The cells were allowed to attach at room temperature (30 min) before the plate was placed on the xCELLigence plate reader in the cell incubator for continuous impedance recording. The real time cell monitoring was conducted at 10°C in an incubator without additional O_2/CO_2 using the RTCA single plate xCELLigence platform. The data were collected at intervals of 2 min after contaminant exposure for 12 h, then every 15 min for 120 h. The cell index (CI) is a parameter derived from the measured cell-electrode impedance data that quantifies the status of the cells (Abassi et al. 2009). Generally, when cells attach onto the electrodes, the CI value increases, while a decrease in CI correlates to cell death. A normalised CI (NCI) at a specific time point is calculated by dividing the CI at that particular time by the CI of a reference time point which is set to 1. The last time-point before compound exposure was used for the normalisation, allowing a more precise comparison of the control versus effect of the different contaminant concentrations tested. The CI values presented here were calculated from 4 replicate values.

Data analysis

GraphPad Prism 6.0 software (GraphPad Software Inc., Palo Alto, CA, USA) was used for the statistical analyses of the xCELLigence response curves using one-way analysis of variance together with a Dunnett's post hoc test ($p<0.05$) to detect treatment variation in contaminant-exposed hepatocytes. Mean±SE were calculated for four replicates (n = 4).

4.4 Biomarkers

4.4.1 Three-spined stickleback - splenic leucocyte immune activities

INERIS

Three-spined stickleback (Gasterosteus aculeatus)

Thirty adult sticklebacks (5.6±0.2 cm, 1.9±0.3 g), from one spawn, were obtained in home husbandry (INERIS, Verneuil-en-Halatte, France). Before experiments, the fish were maintained in a tank (50 L, 19±1°C, 350 µS/cm) with a 14/10 h light/dark cycle for one month. During this period, sticklebacks were fed daily with frozen red mosquito larvae and brine shrimp (3% of body weight/day; Europrix, France).

Leucocyte isolation

Each fish was sacrificed by cervical dislocation, measured and weighed. Spleen tissues were removed under aseptic conditions and gently pressed through sterilised nylon mesh (40 µm, Dutscher) with Leibovitz 15 (L15) medium (Sigma-Aldrich) containing heparin lithium (10 $U.mL^{-1}$, Sigma-Aldrich), penicillin (500 $U.mL^{-1}$, Biochrom AG) and streptomycin (500 $\mu g.mL^{-1}$, Biochrom AG) to obtain leucocyte suspension. Then, leucocytes were adjusted at 10^6 $cells.mL^{-1}$ with Malassez haemocytometer to perform analyses.

Ex vivo exposures

For the *ex vivo* exposures, 30 leucocyte suspensions were used for each test. Each concentration was prepared daily by dilution in methanol and 2% nitric acid. Immediately after the mixture dilution process, for each leucocyte suspension and each mixture, 500 µL of leucocyte suspension were mixed in Micronics (Dutscher) with 2 µL of mixture for each concentration tested. In the same way, 2 µL of solvent was mixed with 500 µL of leucocyte suspension to obtain a solvent control used to check the quality of the leucocyte suspension. All samples (controls and leucocyte suspension mixed with pollutants) were incubated at 4°C for 18 h until analyses.

Innate immune biomarkers analysis

Analyses were carried out on whole leucocytes, using a Cyan™ ADP flow cytometer (Beckman Coulter). For each leucocyte sample, 10 000 cells were counted.

Leucocyte distribution was obtained using FSC and SSC parameters for size and complexity, respectively. Cellular mortality was detected using a double staining method without any inhibitory effect on cellular function (Idziorek et al. 1995). The YO-PRO®-1 (1 mM in DMSO, Invitrogen) and Propidium Iodide (PI, 1.5 mM in water, Invitrogen) were used in order to obtain cellular fluorescence parameters indicating the presence of apoptotic (FL1, green fluorescence) and necrotic (FL3, red fluorescence) leucocytes, respectively (Bado-Nilles et al. 2014). These two markers allow ultrasensitive detection of double-stranded nucleic acids. Nevertheless, activation of P2X7 receptor in apoptotic cells enable penetration of YO-PRO®-1 (Baraldi et al. 2004) in contrast to PI, which is excluded

from viable cells due to their membrane impermeant characteristics. Cell necrosis and apoptosis were detected after 10 min of incubation on ice with YO PRO®-1 (5 µM) and PI (7.5 µM) to limit potential dyes interference with cellular activities, membrane permeability and background staining.

Leucocyte respiratory burst, based on the technique described by (Chilmonczyk et al. 1999), was optimised for the three-spined stickleback. Determination of reactive oxygen species (ROS) in unstimulated cells depends upon the cell incorporating 2'-7'-dichlorofluorescin diacetate (H_2DCF-DA, Sigma-Aldrich), which is a stable non-fluorescent molecule hydrolysed to DCFH by cytosolic enzymes. When leucocytes are stimulated with phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich), the most specific inductor of respiratory burst (Ambrozova et al. 2011; Chadzinska et al. 2012), H_2DCF-DA is hydrolysed by H_2O_2. Finally, the DCFH obtained is oxidised to the fluorescent dichlorofluorescein (DCF) to permit quantification of unstimulated and stimulated cells by flow cytometry. Stimulation index of respiratory burst was determined, after 30 min of incubation at room temperature, as the ratio of fluorescence of PMA stimulated cells (H_2DCF-DA at 60 µM plus PMA at 15 µM) to that of unstimulated cells (H_2DCF-DA at 60 µM).

The lysosomal membrane integrity (LMI) was detected as previously described (Bado-Nilles et al. 2013). Briefly, samples were incubated using AO (10 µM) during 20 min in the dark and at room temperature in order to obtain cellular fluorescence parameters indicating the presence of lysosomes with intact lysosomal membrane (FL3).

The phagocytosis activity was measured using fluorescent microsphere (2.7×10^{10} particles mL^{-1}, Fluorospheres® carboxylate-modified microsphere, diameter 1 µm, Molecular Probes) as previously described (Gagnaire et al. 2006).

Statistical analysis

Results were expressed as means±standard error with n = 30. Verification of normality and of homogeneity of covariance matrices (homoscedasticity) were conducted using respectively the Anderson–Darling test and the Bartlett test on XLStat 2008 (Addinsoft). If values were not normally distributed, the data was log-transformed using $F(x) = \log(x)$, prior to parametric analysis. Finally, a one-way ANOVA was used to assess the effect of each mixture at each concentration in relation to mean solvent control values. The Student Newman-Keuls's test was used for all multiple comparisons. All hypotheses were tested for statistical significance at the level of $p \leq 0.05$.

4.4.2 Atlantic salmon – regulation of molecular biomarkers

NTNU

Exposure

Immature Atlantic salmon (*Salmo salar*, mean weight and length 10±2.5 g and 9±2 cm, respectively) were obtained from Lundamo hatcheries (Trondheim, Norway) and kept in 50 L tanks at 7±0.5°C and for a 14/10 h photoperiod at the Department of Biology, Norwegian University of Science and Technology (NTNU) animal holding facilities. The experiment was performed after 24 hours acclimation period. Five groups of 10 fish were exposed once for 5 days to the chemical mixtures, one group serving as solvent carrier control that was exposed to the carrier vehicle methanol (0.01%) and one blank control. The final concentration of methanol was similar in all exposure groups. During the experimental period, fish were starved and duplicate sampling of 5 fish from each exposure group was sacrificed for gene expression and enzyme activity, respectively, and no fish mortalities were observed. Samples were collected from each exposure group after the fish were anaesthetised with benzocaine (5 mg/L) and blood was collected before sacrifice.

After sacrifice, the liver was excised and weighed. Specifically, 800+800 µl of the reference mixtures were diluted in 50 L water exposure tank and samples were collected at days 3 and 5 after exposure.

ELISA assay

The semi-quantitative vitellogenin (Vtg) ELISA was performed according to standard protocol (Arukwe et al. 1997). Plasma samples were diluted to 1:500 in coating buffer (0.05 M sodium-bicarbonate buffer, pH 9.5). The diluted samples were adsorbed to microtiter wells (overnight at 4°C) and incubated with polyclonal rabbit Arctic char Vtg antibody PO-1 or rabbit anti-salmon Zrp O-146 (diluted 1:2000, Biosense Laboratories) for 1 h at 37°C. Goat anti-rabbit (CYP1A) peroxidase-conjugated secondary antibody (GAR-HRP, Bio-Rad) diluted 1:3000 and H_2O_2/o-phenylenediamine dihydrochloride (OPD) were used for ELISA detection at 492 nm using a Synergy HT microplate reader (Bio-Tek Instruments Inc., Winnoski, Vermont, USA).

RNA purification and cDNA synthesis

Total RNA was purified from liver tissues homogenied in Trizol reagent according to manufacturer's protocol. Total cDNA for the real-time PCR reactions were generated from 1 µg DNase-treated total RNA from all samples using poly-T primers from iScript cDNA Synthesis Kit as described by the manufacturer (Bio-Rad).

Quantitative (real-time) polymerase chain reaction (qRT-PCR)

qRT-PCR with gene sequence primer pair was used for evaluating gene expression profiles. For each treatment, the expression of vitelogenin (Vtg), estrogen receptor alpha (Erα) or zona radiante protein (Zrp) was analysed as described previously (Arukwe, 2005), using the Mx3000P Real-Time PCR System (Stratagene, La Jolla, CA, USA). Each 25 µL DNA amplification reaction contained 12.5 µL of iTAQ™SYBR® Green Supermix with ROX (Bio-Rad), 1 µL of cDNA and 200 nM of each forward and reverse primers (Table 7). Controls lacking cDNA template (minus RT sample) were included to determine the specificity of target cDNA amplification as described previously (Arukwe, 2005). Cycle threshold (Ct) values obtained were converted into mRNA copy number using standard plots of Ct versus log copy number. The criterion for using the standard curve is based on equal amplification efficiency with unknown samples and this is usually checked prior to extrapolating unknown samples from the standard curve. The standard plots were generated using known amounts of plasmid containing the Vtg, ERα or Zrp amplicon.

Table 7. Primers and qRT-PCR programme used to evaluate *Vtg*, *ERα* or *Zrp* gene expression profiles. F: forward. R: reverse.

Primers		**3-step qRT-PCR programme**	
		95°C 5 min	Enzyme activation
F: AAGCCACCTCCAATGTCATC R: GGGAGTCTGTCCCAAGACAA	*Vtg*	95°C 30 sec 57°C 30 sec 72°C 30 sec	40 cycles
F: TCCAGGAGCTGTCTCTCCAT R: GATCTCAGCCATACCCTCCA	*ERα*	95°C 30 sec 55°C 30 sec 72°C 30 sec	

F: TGACGAAGGTCCTCAGGG R: AGGGTTTGGGGTTGTGGT	*Zrp*	95°C 30 sec 55°C 30 sec 72°C 30 sec	
		95°C 1 min 55°C 30 sec	Melting analysis
		55-95°C	Decreasing fluorescence detection with increasing temperature

4.4.3. Genetically engineered bioluminescent organisms

4.4.3.1 Escherichia coli (bacterium)

HUJI

Aqueous exposure of bacterial reporter strain

The reference materials RM040-044 were diluted 1/100 in 200 mM Tris pH 7 to prepare concentrated Mix14 and Mix19.

A panel of 12 engineered bioluminescent microbial reporters (Table 8) was grown overnight in 170 µL LB supplemented with 100 µg/mL ampicillin in a 96-wells plate. The cultures were diluted 100-fold in M9 medium in a 96 deep-well plate (10 µL in 1 mL M9 medium), and regrown with shaking at 37°C for 3 hours. Next, culture aliquots (50 µL) were transferred into an opaque white 96-well microtiter plate (Greiner Bio-One), each well already containing 50 µL of a predetermined concentration of the mixture tested or 50 µL of a predetermined concentration of a model chemical (positive control, Table 8). Each mixture was tested in a concentration series ranging from 0.08× to 5× EQS. Luminescence was measured at 37°C at 10 min intervals using a VICTOR^2 plate reader (Wallac, Turku, Finland). All experiments were carried out in duplicate and were repeated at least 3 times.

Luminescence values are displayed as the instrument's arbitrary relative luminescence units (RLU). Activity was calculated either as the difference in the intensity of the signal in the presence and absence of the inducer (ΔRLU) or as the response ratio, the luminescence in the presence of the inducer divided by that in its absence (Belkin et al. 1997; Belkin, 1998).

Table 8. Bioluminescent *E. coli* reporter strains and their model toxicant.

***E. coli* strain**	**Promoter**	**Type of stress sensed**	**Model toxicant (positive control) and its inducing concentration (mg/L)**	**Reference**
RFM443	*recA*	DNA damage	Nalidixic acid, 10	(Elad et al. 2011)
RFM443	*katG*	Oxidative - peroxides	Hydrogen peroxide, 10	(Yagur-Kroll et al. 2011)
MG1655	*micF*	Oxidative - superoxides	Paraquat, 500	(Elad et al. 2011)
PHL	*zntA*	Excess Cd(II), Pb(II) and Zn(II)	$CdCl_2$, 50	(Kessler et al. 2012)

MG1655	*arsR*	Excess arsenic	Sodium arsenite, 10	(Elad et al. 2011)
DE112	*fabA*	Membrane damage	Phenol, 1000	Lab collection
DE112	*grpE*	General/protein damage	Ethanol, 4%	Lab collection
RFM443	*marR*	Antibiotics and phenolics (oxidative)	Chloramphenicol, 1	(Melamed et al. 2012)
RFM443	*cydA*	Respiratory inhibitors	Na cyanide, 10	Lab collection
MG1655	*sodA*	Oxidative - superoxides	Paraquat, 500	(Kessler et al. 2012)
MG1655	*yqjF*	Specific nitro aromatics	2,4 Dinitrotoluene, 156	(Yagur-Kroll et al. 2013)
MG1655	*soxS*	Antibiotics and oxidative stress agents	Paraquat, 500 or chloramphenicol, 1	(Melamed et al. 2012)

4.4.3.2 Saccharomyces cerevisiae (yeast)

JRC

An increased sensitivity of the yeast-based test system was obtained by using a strain deleted in the multidrug transporters PDR5 (pleiotropic drug response), SNQ2 (disruption confers sensitivity to 4-nitroquinoline *N*-oxide) and YOR1 (yeast oligomycin resistance) (Schmitt et al. 2005). It was previously shown that the deletion of these transporters increases the sensitivity of yeast toward a variety of organic compounds (Schmitt et al. 2004).

For cytotoxicity assessment, growth inhibition assays were performed to determine EC_{50} values. For genotoxic testing, a transcription activation assay, involving the DNA damage inducible RAD54 promoter fused to the yeast enhanced version of the green fluorescent protein of *Aequorea victoria* (Cormack et al. 1996), was used as a genetic marker for general toxicant-inducible DNA integrity damage. Thereby the induction of green fluorescence serves as the genotoxic endpoint.

For toxicant-induced stress, a destabilised version of the green fluorescent protein (GFP) optimised for expression in yeast under control of the promoter of the housekeeping plasma membrane ATPase gene *PMA1* was used. *PMA1*, one of the most prominent housekeeping genes in *S. cerevisiae*, encodes the major plasma membrane H^+-ATPase (Serrano et al. 1986) and is essential for viability. A decrease in membrane potential has been suggested as primary cellular stress signal triggering the intracellular response (Moskvina et al. 1999). The PPMA1-mediated transcriptional activation of the yeast optimised green fluorescent protein (yEGFP3) results in the production of the green fluorescent protein. To monitor dynamic fluorescent changes in the assay, a fluorescence emission decrease indicates dose-dependent intoxication. To that end, a destabilised version of GFP is constructed by coupling the PEST-rich C-terminal residues of the G1 cyclin *CLN2* which, as universal ubiquitin targeting sequence, confers rapid degradation of yeast proteins (Mateus and Avery, 2000). The shift of the steady-state turnover of PPMA1-driven GFP transcription and PEST-mediated degradation upon intoxication, toward less transcription/translation and thus proportionally increased degradation, led to decreased fluorescence that served as a reporter for toxicant-induced stress.

Strains and growth conditions

The *Saccharomyces cerevisiae* strains used in this study are listed in Table 9.

Yeast cells were grown on minimal YNB medium (per 1 L of final solution): 1.7 g yeast nitrogen base (without amino acids and without (NH4)2SO4), 10.5 g citric acid buffer, and 0.5 g amino acid drop out mix (41.7 mg adenine, 83.3 mg tryptophane, 16.7 mg arginine, 16.7 mg methionine, 25 mg tyrosine, 25 mg lysine, 50 mg valine, 83.3 mg threonine, 83.3 mg serine, 41.7 mg phenylalanine, 16.7 mg asparagine, 16.7 mg glutamic acid). The pH was adjusted to 6.4. After autoclaving (120°C, 20 min), 12.5 mL of a 40% glucose solution (final concentration: 0.5%) and 10 mL of a sterile solution of L-leucine (4 mg/mL) and histidine (2 mg/mL) were added to the growth medium. YNB was purchased from DIFCO, all amino acids, 4-nitroquinoline-*N*-oxide (4-NQO), and *N*-methyl-*N*-nitrosourea (NMU) were obtained from Sigma-Aldrich. All components were of analytical grade.

Table 9. Yeast strains used in the study of toxicant-induced stress.

Name	Plasmid	Relevant Genotype	Origin
FYAK26/8-10B1 (sensitive mutant, parental strain	-	MATa ura3-52 trpΔ63 leu2Δ1 his3Δ200 GAL2+ pdr5Δ1::hisG snq2::hisG yor1::hisG	(Kolaczkowski et al. 1998)
#261 (indicator strain: genotoxicity)	pY-$P_{RAD54\text{-}s}$-yEGFP3	As FYAK26/8-10B1 plus [$P_{RAD54\text{-}s}$-yEGFP3 URA3 LEU2]	AG Molecular Bioenergetics
#545 (control strain: genotoxicity)	pY-$P_{RAD54\text{-}s}$-yEGFP3Δ	As FYAK26/8-10B1 plus [$P_{RAD54\text{-}s}$ URA3 LEU2]	AG Molecular Bioenergetics
#544 (indicator strain: acute toxicity)	pY-P_{PMA1}-yEGFP3/PEST	As FYAK26/8-10B1 plus [P_{PMA1}-yEGFP3/PEST URA3 LEU2]	AG Molecular Bioenergetics
#549 (control strain: acute toxicity)	pY-P_{PMA1}-yEGFP3	As FYAK26/8-10B1 plus [P_{PMA1}-yEGFP3 URA3 LEU2]	AG Molecular Bioenergetics

Assay conditions and fluorescence monitoring

Stock cultures, preserved in solid selective medium, were freshly grown in liquid medium, and incubated overnight at 30°C agitation at 250 rpm. For the tests, cells were harvested by centrifugation and resuspended in fresh medium to a final concentration of 10^6 cells/mL, corresponding to an optical density (OD) of 0.25 AU (Eppendorf Biophotometer Plus).

In all tests, 96-well microtiter plates were used with an inoculum of 200 µL per well ($2x10^5$ cells/well). The cultures were incubated at 30°C with continuous agitation at 120 rpm. To avoid evaporation during the incubation period, plates were sealed with adhesive film.

Each experiment consisted of a minimum of eight different compound concentrations (determinations were performed in eight replicates), including the following controls: (a) negative control cultures (YNB medium with inoculum and 10 µL of solvent) to determine maximum proliferative capacity and unimpaired fluorescence intensity; (b) blank controls (compounds in appropriate concentrations to determine endogenous compound absorbance and fluorescence; (c) YNB medium to monitor potential contamination; and

(d) positive control cultures (reference toxicant, e.g. 4-NQO) to ensure that the test and/or its components produced reliable results. For each tested chemical at least sixteen replicates were carried out on different days.

For growth and genotoxicity tests, plates were incubated for 8 h while for acute toxicity tests they were incubated for 4 h. Results were considered as valid only if the turbidly of the negative control cultures increased at least four times during the test period. Fluorescence (λ_{ex} 485 nm, λ_{em} 535 nm) and turbidity (OD 600 nm) were measured (Tecan Spectra Fluoro Plus) at time zero (start of the experiment) and at the end of the incubation period.

Chronic toxic effects were calculated by quantifying the extent of growth inhibition in the test cultures in comparison with the control cultures after the incubation time (mean growth of 16 replicates per test condition).

Growth inhibition (G_i) was calculated as follows: $G_i = (G_C - G_T) / G_C) \times 100$ (where G_C is the arithmetic mean of OD 600 nm of the control cultures at the end of the incubation period corrected for the starting OD, G_T is the arithmetic mean of the OD 600 nm of the test cultures at the end of the incubation period corrected for the starting OD.

Fluorescence was measured by the induction ratio (IR) calculation. The IR is the ratio of the arithmetical mean fluorescence (corrected for blanks) divided by growth determined as the OD 600 nm (also corrected for blanks) at the test concentration normalised to the corresponding values for the negative control cultures. That is: $IR=1/G_i \times C_i/C_n$ (where C_i is the mean fluorescence corrected for blank of the test concentrations at the end of the incubation period, C_n is the mean fluorescence corrected for blank for the negative controls at the end of the incubation period and G_i represents the mean inhibition of growth) as indicated in Schmitt et al. (2004).

The yeast cultures were exposed to 2 different mixtures, Mix14 10X EQS and Mix19 1X EQS at different concentrations ranging from 1- to 250-fold EQS-eq for Mix14 10X EQS, and from 0.5- to 25-fold EQS-eq for Mix19 1X EQS. Previous to the mixture evaluation, the number of cells per well were optimised to maximise fluorescence response as well as solvent volume. Positive controls were also tested and they ranged from 1 to 1000 µg/L for 4-nitroquinoline-1-oxide (4-NQO), from 1 to 5000 µg/L for *N*-nitroso-*N*-methylurea (NMU) and from 10 to 5000 µg/L for cadmium (Cd).

Data were considered not reliable when Gi was >20% (Schmitt et al. 2004). For genotoxicity, a chemical was considered genotoxic when IR >1.4.

4.4.3.3 Caenorhabditis elegans (nematode)

AESD

Strains

The growth size, pharyngeal pumping, movement and Nile red assays were performed with wild type (N2 Bristol) nematodes. The *in vivo* nematode fluoromics assay utilised five transgenic strains (all created by Knudra, UT, USA): *cyp-35A2* (58cop (25.3.47)), *mtl-2* (62cop (6.15.47)), *ugt-1* (59cop (8.13.47)), *gst-38* (54cop (7.7.47)) and *gcs-1* (23cop (5.23.47)). Each transgenic strain was dual-labelled, namely by linking the promoter of the biomarker to the coding region of a red fluorescent protein (mCherry) and an invariant transmembrane vesicular γ-aminobutyric acid (GABA) transporter, *unc-47,* to the coding region of a green fluorescent protein (GFP). All strains were maintained at 20°C on nematode growth medium (NGM) agar plates which were seeded with *Escherichia coli* (OP50).

NGM-Plate preparation and exposure conditions

The exposure mixtures (Mix14 and Mix19) and the solvent control were prepared from solutions RM040-RM044 as instructed and added to OP50 to generate final dilutions of 1:1000 and 1:100. Standard bacterial growth assays confirmed that the supplementation of the exposure mixtures did not influence the growth of the *E.coli* OP50 (data not shown). In addition, two positive controls were used in the fluoromics experiments, namely BaP (100 µg/mL) for *cyp35A2* and $CdCl_2$ (100 µM) for *mtl-2*. NGM agar plates (20 mL volume) were inoculated with 200 µL of the spiked OP50 and the seeded plates were incubated at room temperature for 24 h (to allow for bacterial growth). All strains were age-synchronised, placed (as L1 larvae) on the NGM-plates and exposed to the respective conditions for 48 h at 20°C. For Nile red experiments, a stock solution was diluted in OP50 (final concentration: 2 µg/mL) and added to the appropriate reference mixtures. The resultant mixtures were spread on NGM plates and incubated for 24 h at room temperature. As with the other assays, staged nematodes were exposed to each mixture for 48 h (but this time supplemented with Nile red).

Imaging fluoromics and Nile red assays

Single worms were picked onto a glass slide with a drop of M9 and immobilised with sodium azide (2%). Images were captured with a Nikon DS-2Mv digital camera and NIS-Elements F 2.20 software linked to a Nikon ECLIPSE TE2000-S inverted microscope. The fluorescence intensities from 10 worms per condition were analysed using ImageJ. The following filters were used to quantify the fluorescence: G-2A (Ex 510-560nm) for mCherry and Nile red, and FITC (Ex 465-495nm) for GFP.

Pharyngeal Pumping Food intake

Pharyngeal pumping was assessed in N2 nematodes (subjected to the above mentioned reference mixtures) by counting pharyngeal bulb contraction over a time period of 2x30 sec. Pumping was assessed by means of high magnification Apochromatic Zoom and FusionOptics™ microscopy (Leica M205C) and quantified from 15 nematodes per condition.

Growth Size Assay

Wild type nematodes (n = 10 per condition) were plated on NGM plates (containing the OP50 and the different refrence mixtures) and maintained for 48, 72, 96 and 120 h. Adult nematodes were transferred to new plates between 72 and 96 h to remove hatched offspring. Images of worms were obtained using an inverted microscope (as described above) and the flat volumetric surface area and length determined by tracing the nematodes using the Image-Pro Express software (Media Cybernetics, Inc.).

Movement Assay

The movement of wild type nematodes (challenged with the respective reference mixtures) was assessed after 48, 72 and 96 h by determining the distance travelled on agar within a 30 sec timeframe. Movement was quantified from 15 nematodes per condition using the Image-Pro Express software (Media Cybemetrics, Inc.).

Statistical analyses

Data obtained from the fluoromics and Nile red staining were analysed using the one-way ANOVA followed by the Tukey's multiple comparison test to test for significant differences

between the reference mixtures. The phenotypic assays were assessed by means of the two-way ANOVA. All tests were executed with GraphPad Prism.

4.4.4 Quantitative real-time PCR (qRT-PCR)

ORU

Cell lines, maintenance and exposure

Cell lines were obtained from ATCC and maintained according to provider's recommendations. The human epithelial cervix HeLa cell line was cultured in DMEM (HyClone) + 10% FBS (HyClone) (Carvalho et al. 2014). The chicken epithelial hepatocellular LMH cell line was cultured in Waymouth´s MB (Gibco) + 10% FBS in gelatin coated (0.1% gelatin from porcine skin, Sigma-Aldrich) flasks or wells (Asnake S et al. 2013). Both cell lines were incubated at 37°C at 5% CO_2. Zebrafish epithelial liver ZFL cell line was cultured in 50% L-15 (Gibco), 35% DMEM High glucose (PAA Laboratories), 15 % Ham´s F12 (Gibco) supplemented with 5% FBS, 15 mM HEPES, 0.15 g/L sodium bicarbonate, 1X insulin-transferrin-selenium (Gibco)) at 28°C and 3% CO_2 (Lungu-Mitea et al. 2018).

Exposure mixtures were reconstituted in Milli-Q water and immediately before use mixed with cell culturing medium (1 + 4) to get the desired exposure concentration (Mix14 at 1X EQS and 10X EQS, and Mix19 at 1X EQS). That mix did not affect the pH of the cell culturing media. Prior to exposure, cells were plated in 6- or 12-wells plates, and after 18-20 hours exposed to the mixtures. HeLa and LMH cells were treated for 24 hours and ZFL for 40 hours, n = 4. The chemical mixtures diluted in water were 1 day old when ZFL cells were exposed, 2 days for LMH and 3 days for HeLa cells.

RNA extraction and qRT-PCR

Following exposure the cells were lysed and total RNA was isolated using NucleoSpin RNA II kit (Macherey-Nagel, Germany) according to manufacturer's manual, RNA was quantified by Nano-Vue (GE Healthcare, USA) and thereafter stored at -80°C. cDNA synthesis was prepared using qScript cDNA synthesis kit (Quanta Biosciences, USA) and qRT-PCR was performed using KAPA SYBR FAST qPCR kit (KapaBiosystems, USA) according to manufacturer's recommendations on Mx 3000P qPCR system (Stratagene, USA). The following thermocycling conditions were used: denaturation 5 min at 95°C followed by 40 cycles of 95°C for 2 seconds and 60°C for 30 seconds (Pradhan A et al. 2017). The obtained Ct values were normalised using elongation factor 1 alpha 1 (*eef1a/1*) and relative gene expression was determined by the ΔΔCt method (Schmittgen and Livak, 2008). Primers used and their target genes are listed in Table 10.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, USA) by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test for multiple group comparison and significant differences were considered if the p values were <0.05 (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

Table 10. List of primers used for qRT-PCR and the target genes. F: forward. R: reverse.

Cell type	Gene symbol	Gene name	Primer (5'-3')
HeLa	*EF1a*	Elongation factor 1-alpha	F: TCTGGTTGGAATGGTGACAA R: ACGAGTTGGTGGTAGGATGC

	AR	Androgen receptor	F: GCGCCAGCAGAAATGATTGCACTA R: ACACTGTCAGCTTCTGGGTTGTCT
	ERα	Estrogen receptor alpha	F: ACACATTTCTGTCCAGCACCCTGA R: CACCACGTTCTTGCACTTCATGCT
	ERβ	Estrogen receptor beta	F: TGGAGTCTGGTCGTGTGAAG R: GTCGGCACTTCTCTGTCTCC
	MT2A	Metallothionein 2A	F: AGGGCTGCATCTGCAAAG R: GGTCACGGTCAGGGTTGTA
	CYP1A1	Cytochrome P450, family 1 subfamily A, polypeptide 1	F: AGTGGCAGATCAACCATGACCAGA R: TGCATTTGGAAGTGCTCACAGCAG
	GSTK1	Glutathione S-transferase kappa1	F: ATCCAGAGATGCTGGAGAAAGCGT R: CTTCACCTTTGGCGTTGCGATCTT
	COX2	Cyclooxygenase-2	F: AGGGTTGCTGGTGGTAGGAATGTT R: AAGTGCTTGGCTTCCAGTAGGCAG
	IL-6	Interleukin-6	F: GCAGAAAAAGGTGGGTGTGT R: GCAGAAGAGAGCCAACCAAC
	IL-8	Interleukin-8	F: CAGGAATTGAATGGGTTTGC R: AAACCAAGGCACAGTGGAAC
	p53	Tumor supressor protein p53	F: GTCTTTGAACCCTTGCTTGC R: CCACAACAAAACACCAGTGC
LMH	*EF1a*	Elongation factor 1-alpha	F: CTGGATTGCCACACTGCTCACA R: GATTTCAGGAACTTCGGGCCATCC
	AR	Androgen receptor	F: ACGAGTACCGGATGCACAAATCCA R: TTCTGGTTCTTCAGGCCATCCACT
	ESR1	Estrogen receptor 1	F: TTTCCAGTGCTCACCCTGCATTTG R: AGTCTCCAGCTCAGTGCCTTGAAT
	ESR2	Estrogen receptor 2 (beta)	F: ACGCACACCTCTGTCTGTTTCTGA R: TCTTGCAGGACTGTTCTGAGGCTT
	MT	Metallothionein	F: GCACGTGTGGAGACAACTGCAAAT R: ACAGCCCTTGGCACAGTTGTT
	CYP1A1	Cytochrome P450, family 1 subfamily A, polypeptide 1	F: GAGTTTGACCTTCAGCACCGACAC R: TCGAAGCTCTGCTTCTCCTCCATC
	GST	Glutathione S-transferase	F: GCACGTGTGGAGACAACTGCAAAT R: ACAGCCCTTGGCACAGTTGTT
	COX2	Cyclooxygenase-2	F: GCCTACTAGAAGTCGACCATCGCA R: ACTCCTGGTCGAGTGGTGATGAAG
	IL-8	Interleukin-8	F: GATCCCTTGGAAGCCACTTCAGTC R: GTCGGCATGAGCTGACTCTGACTA
	p53	Tumor supressor protein p53	F: CCTGCTTGATGGACGAGAGTTGGT R: TGGTGACGTAGACGGACATGCT
ZFL	*EF1a*	Elongation factor 1-alpha	F: GTACTACTCTTCTTGATGCCC R: GTACAGTTCCAATACCTCCA
	AR	Androgen receptor	F: ACAACACACCTGGATGGGAGTGAT R: TGACCTGTAGCAGCACAAACTCCT
	ESR1	Estrogen receptor 1	F: AAGAACTCGTCCACATGATCGCCT R: AGACTCCGAAATCGAGCCACAGTT

	ESR2a	Estrogen receptor 2a	F: TACGACTTCAGCACTCTGCCCTTT R: CCGCTTTACCAGTGGTTTGCTGTT
	ESR2b	Estrogen receptor 2b	F: TGTTCGAGTTTGCCACAGACTCCT R: ACAGATGCTCGATGCCTTTGTTGC
	MT2	Metallothionein 2	F: CCTGCAAGTGCACTAATTGCCAGT R: ACGCAGACGTGGAGTAGACAAACA
	CYP1A	Cytochrome P450, family 1 subfamily A	F: AGGCTGGTGATGGAGCATTACGAT R: ATCGGACACTTGCAGGTTGGAGTT
	MGST1	Microsomal glutathione-S-transferase 1	F: GCACTTCCGGGTGTTTGTAGTGTC R: GTGAGCACCCTGTAGGCCATAGAT
	COX2	Cyclooxygenase-2	F: CACTGTTGCCGGACAACTTTCAGA R: TCCAGCAGTCTGTTTGGTGAAGGA
	IL-8	Interleukin-8	F: CAGGTGATCCGGGCATTCATGGT R: AATGAGCTTGAGAGGTCTGGCTGT
	p53	Tumor supressor protein p53	F: AGTTAAGTGATGTGGTGCCTGCCT R: ATCAGCTTCTTTCCCTGTTTGGGC

4.4.5 Receptor-binding

4.4.5.1 Yeast Estrogenic Screen (YES) assay

ECOTOX

In the YES assay the recombinant yeast cell *Saccharomyces cerevisiae* containing the human estrogen receptor α (hERα) is exposed to samples in 96-well plates. The receptor activity is indicated by the reporter gene *lacZ* encoding β-galactosidase, which metabolises the CPRG (chlorophenolred-β-galactosidase) to CPR (chlorophenolred) and so resulting in a color shift from yellow to red. After 72 h of exposure the receptor activity respects the color changes and the cell growth for the correction is measured in a microplate absorbance reader (540 nm and 620 nm). This receptor binding system enables capturing the combined effect of compounds with estrogenic activity such as 17β-estradiol, 4-nonylphenol, bisphenol A, fluoranthene, etc. 17β-estradiol is used as reference compound and the results are expressed as EC_{10} and EC_{50} (the concentration causing 10% and 50 % of the maximum effect, respectively) aswell as EEQ (estrogen equivalent concentration). The evaluation of the generated data by fitting a dose-response curve was carried out with GraphPad Prism 5 Software (La Jolla, CA, USA). The fit provided the EC_{50} value and out of this the EC_{10} and EEQ values were calculated.

4.4.5.2 CALUX bioassays

ECOTOX

The samples were analysed with three different CALUX bioassays:

- Estrogen receptor (ER)-CALUX method is a sensitive biological detection method for estrogens and natural and synthetic estrogen-like compounds.
- Androgen receptor (AR)-CALUX method is a sensitive biological detection method for androgens, anabolic steroids and natural and synthetic androgen-like compounds.
- PPAR-CALUX method is a sensitive biological detection method for measuring peroxisome proliferator-activated receptor ɣ2-like (PPARɣ) activity. PPARɣ ligands are thought to be of importance with respect of "obesogens", agents that inappropriately

regulate and promote lipid accumulation and adipogenesis (Grün and Blumberg, 2009).

All CALUX bioassays used utilise the U2OS human cell line with a luciferase gene under the transcriptional control of responsive elements for activated hormone receptors. The receptor varies depending on the endpoint of the assay.

In short, cells were seeded into 96-well plates with DF medium (without phenol red) that was supplemented with stripped (dextran-coated charcoal treated) serum. After 24 h of incubation (37°C, 7.5% CO_2), the medium was replaced by medium containing the reference mixtures extracts (1% DMSO) for agonistic activity testing and after further 24 h of incubation, the medium was removed and the cells were lysed in 30 µL of Triton lysis buffer. The amount of luciferase activity was quantified using a luminometer (MicroLumat Plus, Berthold Technologies, Switzerland). On all plates, a dose-response curve of the reference compound was included for quantification of the response, which was estradiol, dihydrotestosterone or rosiglitazone for the ER- (Van der Linden et al. 2008), AR- (van der Burg et al. 2010) or PPAR-CALUX (Gijsbers et al. 2011), respectively. All extracts and reference compounds were analysed in triplicates. Only dilutions that were negative in the cytotoxicity test were used for quantification of the response (Van der Linden et al. 2008).

The evaluation of the generated data by fitting a dose-response curve was carried out with GraphPad Prism 5 Software (La Jolla, CA, USA). The results were expressed as EC_{50} (the concentration causing 50% of the maximum effect) as well as EEQ (estrogen equivalent concentration), DHT-EQ (dehydrotestosterone equivalent concentration) or REQ (rosiglitazone equivalent concentration), respectively.

4.4.5.3 ER-, AR- and PXR-activated luciferase induction

INERIS

The ER-, PXR- and AR-mediated activities of the mixtures were monitored by using the MELN, HG5LN-PXR and MDA-kb2 reporter cell lines, respectively. The MELN cell line was obtained by stable transfection of MCF-7 human breast cancer cells by an ERE-βGlob-Luc-SVNeo plasmid (Balaguer et al. 2001). The HG5LN-PXR cell line was derived from HeLa cells that were first stably transfected with GAL4RE5-bGlob-Luc-SV-Neo (HG5LN cells) before being stably transfected with the pSG5-GAL4(DBD)-hPXR(LBD)-puro plasmid (Lemaire et al. 2006). The MDA-kb2 cell line derived from the MDA-MB-453 human breast cancer cells that were stably transfected by a MMTV promoter-luciferase plasmid construct under the control of endogenous AR and glucocorticoid receptor (GR) (Wilson et al. 2002). All reporter cell lines were routinely cultured in phenol red containing Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 5% foetal calf serum (FCS) at 37°C under 5% CO_2 humidified atmosphere.

Cells were seeded into 96-well plates at a density of 50000 cells/well in phenol red-free DMEM supplemented with 3% stripped serum. Twenty-four hours after plating, cells were dosed with various dilutions of mixtures, solvent control and positive control. Before reconstitution of mixtures, RM040, RM041 and RM042 have been first concentrated under N_2 evaporation to allow testing Mix14 and Mix19 in a broader concentration range. The final concentration of methanol/citric acid solvent was always 0.5% v/v (0.25% each). At this concentration, no solvent effect was noted in any of the assays. All tests included a dose-response curve of the reference compound, namely estradiol, dihydrotestosterone and SR12813 for MELN, MDA-kb2 and HG5LN-hPXR respective assays. Upon overnight exposure (18 h), 0.3 mM of D-luciferin was added to the wells. After 5 minutes, the luminescence signal was measured in living cells for 2 seconds per well using luminometer (µBeta, Wallac).

Modeling of dose-response curves and determination of efficient concentrations (i.e. EC_{10} and EC_{50}) were done using the Regtox Microsoft Excel™ macro based on Hill equation model (Vindimian et al. 1983).

RECETOX

Effects on androgen receptor (AR) were evaluated with MDA-kb2 human breast cancer cell line cultivated in Leibowitz L-15 medium (Sigma-Aldrich) supplemented with 10% (v/v) FCS Mycoplex. Exposures were done in Leibowitz L-15 medium supplemented with 5% (v/v) stripped FCS. Cells were seeded into sterile 96-well plates at the density of 50000 cells/well and kept at 37°C without addition of CO_2. For antagonist studies, medium was supplemented 0.5 nM dihydrotestosterone (DHT). Cells were then exposed to a dilution series of the mixtures Mix14 and Mix19. After 24 h exposure, lysis buffer was added to the cells, plates were shaken for 30 min and 100 µL of substrate for luciferase was added with a following measurement of anti/androgenic potency.

4.4.5.4 EASZY - detection of Endocrine Active Substances acting through ERs, using transgenic cyp19a1b-GFP zebrafish embryos

INERIS

The mixtures have been tested in an *in vivo* mechanism-based fish assay that uses transgenic zebrafish *cyp19a1b*-GFP embryos expressing GFP under the control of the zebrafish *cyp19a1b* promoter. This assay is named EASZY (Detection of Endocrine Active Substances acting through ERs, using transgenic *cyp19a1b*-GFP zebrafish embryos) and is under the validation process at OECD.

The *cyp19a1b* gene codes for the brain aromatase which is responsible for the biosynthesis of estrogens, and is strictly regulated by estrogens. Previous works have shown that GFP 1) faithfully mimics the endogenous expression of the *cyp19a1b* gene and 2) is strongly and rapidly induced when embryos are exposed to compounds that activate the ER-signalling pathways within the central nervous system of developing embryos (Brion et al. 2012).

Newly fertilised zebrafish eggs were exposed to test mixture effects on fertilisation for 96 hours under static conditions, i.e. water was not renewed during the exposure period. For each mixture, a range of dilutions was tested. For Mix14, the concentrations tested were 4×, 1.2×, 0.4× 0.12× and 0.04× EQS. For Mix19, the concentrations tested were 0.4× 0.12× and 0.04× EQS.

In each experiment, 17α-ethinylestradiol (EE2) was assessed as a positive control at a final concentration of 0.05 nM. Each mixture was tested at the occasion of three independent experiments performed in March, August and September 2013.

At the end of each experiment, the fluorescence of each living zebrafish embryo was acquired through a fluorescence microscope. The intensity of fluorescence was then quantified using image analysis software (ImageJ). To determine the estrogenic activity of each mixture, the data (expressed as mean fold induction above control) were analysed with a parametric two-way ANOVA and post hoc test using R software.

4.4.5.5 In vitro human ERα^{LBD} competition assay

JRC

The in vitro human estrogen receptor ligand binding domain (ERα^{LBD}) competition assay is suitable for screening the binding ability of chemical substances to estrogen receptor (Ferrero et al. 2014).

The recombinant ERα^{LBD} (Figure 3) was expressed in *Escherichia coli*, purified and used to evaluate the binding affinity for 17β-estradiol, Mix14 and Mix19 using PolarScreen^{TM} ERα Competitor Green Assay (Life Technologies) (Figure 4). The full length ERα provided with the kit was replaced with the recombinant ERα^{LBD} to form the receptor-Fluormone^{TM} complex following incubation with Fluormone^{TM} at 4°C for 45 minutes. The complex was then mixed with Mix14 or Mix19 and incubated at 25°C for 2 h. The following dose-response concentrations were tested: 0.01x to 200x EQS for Mix14 and 0.001x to 200x EQS for Mix19 using 17β-estradiol as reference compound. The intensity of the polarisation signal was calculated from values obtained using Infinite 200 Pro multimode plate reader from Tecan (Ferrero et al. 2014). The data were fitted to a logistic curve using OriginPro Software.

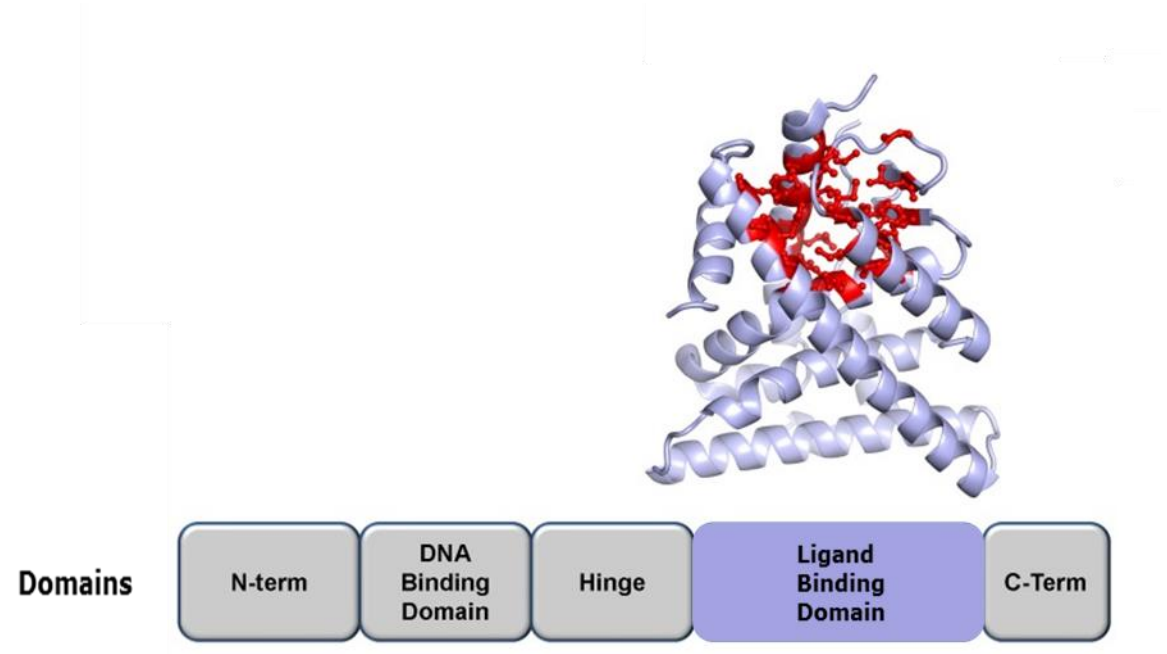


Figure 3. Schematic structure of the estrogen receptor. Domains composing the proten are represented along with three-dimensional structure of the ligand binding domain (LBD) including the ligand binding pocket highlighted in red (form Ferrero et al. 2014).

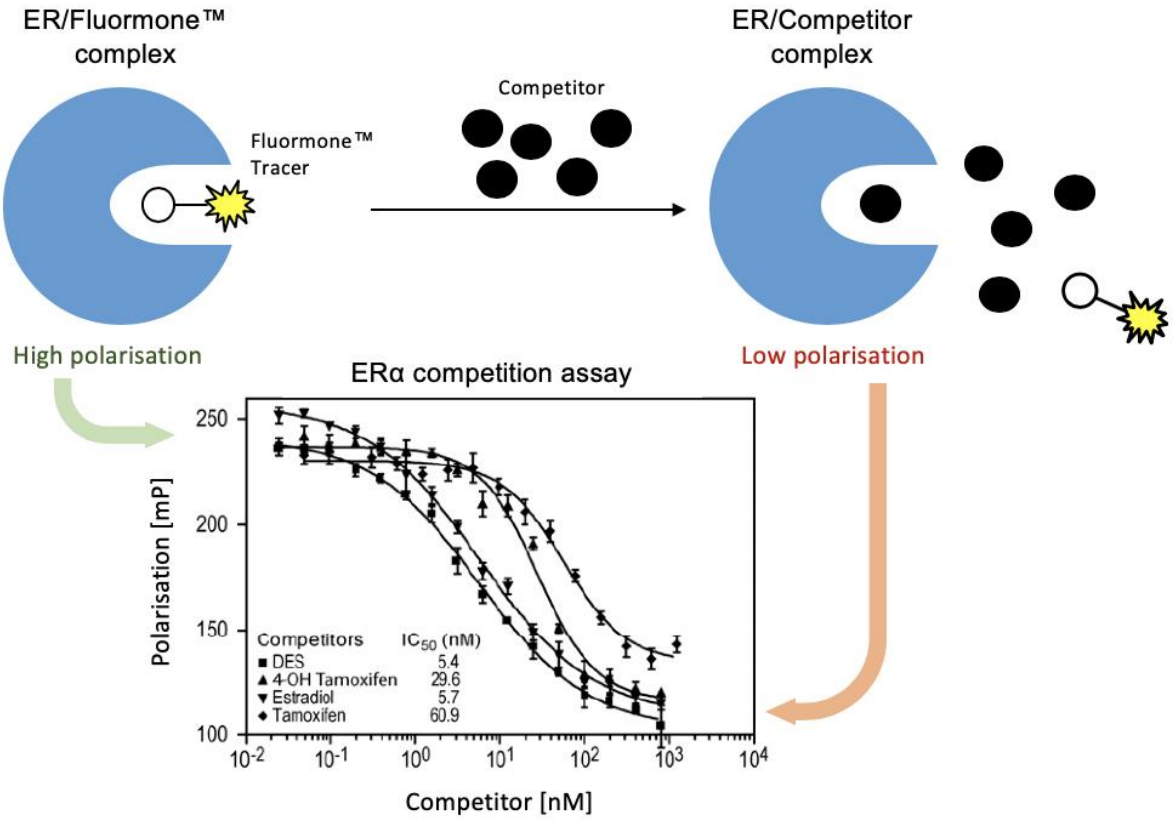


Figure 4. Competitive binding of ligand and fluorescent complex in the ERα pocket. When the receptor binds to the Fluormone™ ligand, which is a biologically relevant estradiol derivative designed to achieve nanomolar binding affinity, the resulting complex yields a high polarisation value. If the test compound (competitor) displaces the Fluormone™ ligand from the complex, the polarisation value decreases. The concentration of the competitor that results in a half-maximal shift in polarisation value equals the IC_{50} of the competitor directly proportional to its relative affinity for the ERα ligand binding domain. Examples of polarisation for four test compounds are shown (adapted from ThermoFisher Scientific).

4.4.5.6 Dioxin-like induction of EROD activity in PLHC-1 cells

INERIS

The fish hepatic PLHC-1 cell line (ATCC, #CRL-2406) was routinely grown at 30°C in E-MEM culture media supplemented with 10% FCS and 1% antibiotics in a 5% CO_2 humidified atmosphere. For experiments, cells were seeded in 96-well plates at a density of 50000 cells per well. After 24 hours of incubation, cells were exposed to various dilutions of mixtures, solvent control and TCDD (2,3,7,8-tetrachlorodibenzodioxin) as positive control for 4 h and 24 h, in order to differentiate between active compounds that are rapidly metabolised (e.g. PAHs) in the cells and dioxin-like chemicals that are persistent in the cells (e.g. dioxins) (Louiz et al. 2008). Then, plates were processed for 7-ethoxyresorufin-O-deethylase (EROD) activity in living cells, as previously described (Louiz et al. 2008).

4.4.5.7 AhR binding-luciferase induction in H4IIE-luc cells

RECETOX

The H4IIE-luc, rat hepato-carcinoma cells stably transfected with the luciferase gene under control of the arylhydrocarbon receptor (AhR) were used for analysis of dioxin-like activity

of the samples. The H4IIE-luc were plated in DMEM-F12 with phenol red (Sigma-Aldrich, USA) containing 10% fetal calf serum and the density of cells were 15000 per well. 24 h after plating into the 96-well plates, the cells were exposed to samples (at least 5 different concentrations of each mixture were tested) with further calibration of the reference compound (TCDD), blank and solvent controls (0.5% v/v methanol maximum). Exposures were conducted in three replicates for 24 h at 37°C. After exposure, intensity of the luminescence was measured using Promega Steady Glo Kit (Promega, Mannheim, Germany).

5. RESULTS

5.1 Effect on the algal-bacterial composition in marine microcosm

JRC

The exposure of the marine microcosm to Mix14 1X EQS and 10X EQS, and Mix19 1X EQS, in seawater resulted in chlorophyll *a* concentration decrease (Figure 5) for all the mixtures. On the other hand, the microcosms exposed to all mixtures resulted in bacterial growth comparing with the untreated control (seawater only) and the solvent control (seawater with methanol and acid) as seen in Figure 6, particularly for Mix14 at 10X EQS and Mix19 at 1X EQS. Figure 7 shows the effect on additional pigments that have been measured.

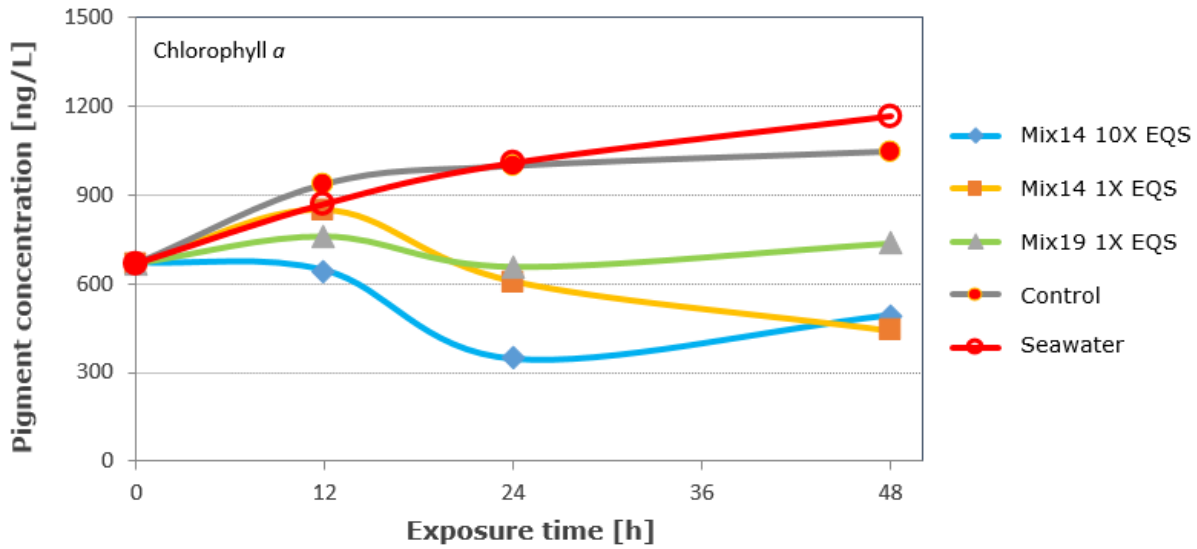


Figure 5. Effect of the chemical mixtures on the phytoplankton community. The graph shows the chlorophyll *a* concentrations in time-dependent exposures of seawater to the mixtures Mix14 at 1X EQS and 10X EQS, and Mix19 at 1X EQS, to the solvent control (SC) and the untreated seawater. For comparison, identical seawater samples were left untreated (SW) or exposed to solvent control (SC).

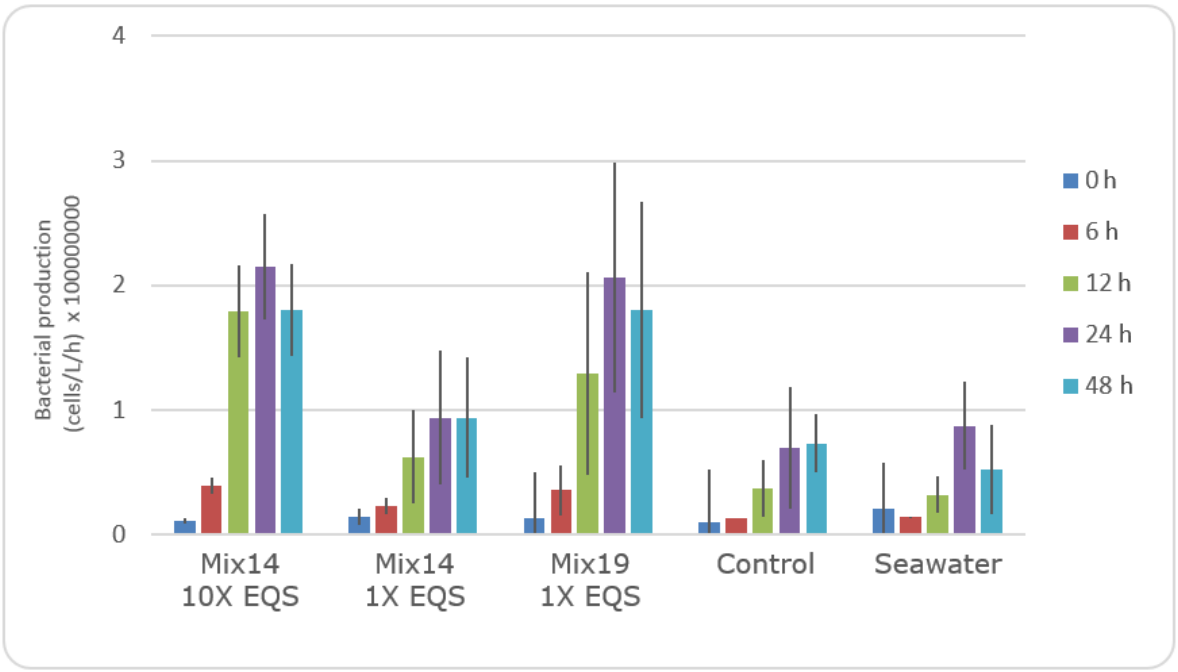


Figure 6. Effect of the chemical mixtures on the bacterioplankton community. The graph shows the comparison between the bacterial production of seawater samples exposed to the mixtures Mix14 at 1X and 10X EQS, and Mix19 at 1X EQS, at different time-points with the exposure to the solvent control (SC) and the untreated seawater.

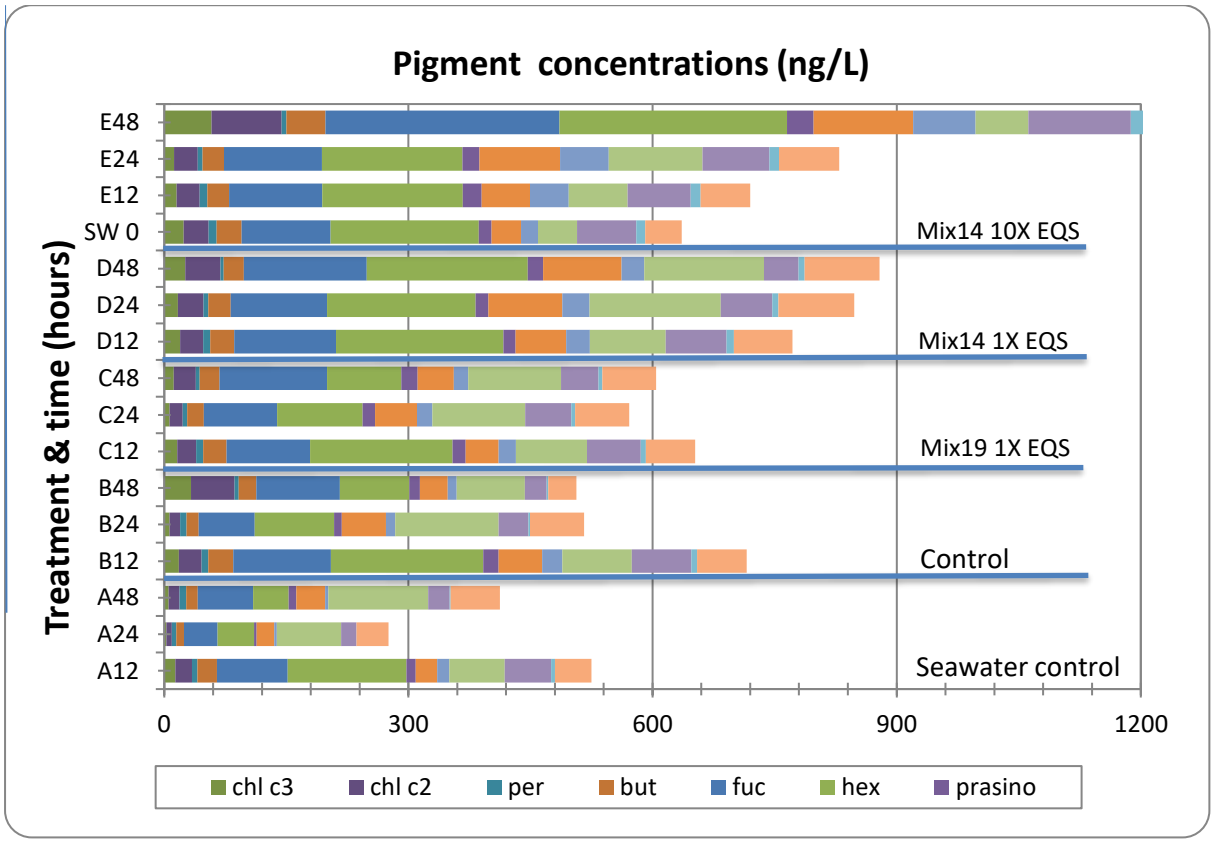


Figure 7. Effect of the chemical mixtures on the presence of pigments in the marine microcosm. In addition to chlorophyll *a* displayed in Figure 5, other pigments were measured for the different exposures, including chlorophyll *c*3 (chl c3), chlorophyll *c*2 (chl c2).

5.2 Acute toxicity

5.2.1 *Vibrio fischeri* - Microtox®

A toxicity effect with the Microtox® test is usually demonstrated by inhibition of luminescence in the bioluminescent bacteria *V. fischeri*. The test was performed in four different laboratories and in all of them no inhibition of luminescence was observed. On the contrary, all the mixtures and the solvent control induced a slight increase in the bacteria luminescence.

RECETOX

For the three mixtures, a stimulation of luminescence was observed. Therefore, no toxicity effect was induced by exposure of *V. fischeri* to any of the reference materials (Mix14 1X and 10X EQS, and Mix19 1X EQS) nor the solvent control (Table 11).

Table 11. Effect of the studied samples at 1x concentration (no enrichment) in the Microtox® test with *V. fischeri*.

Sample	Effect on luminescence	
	15 min	30 min
Mix14 10X EQS	20% increase	36% increase
Mix14 1X EQS	18% increase	35% increase
Mix19 1X EQS	22% increase	39% increase

NOTE: very low stimulation of luminescence in solvent control, statistically not significant

ECOTOX

Table 12 and Figure 9 present the effect data based on and derived from the nonlinear fit generated by GraphPad Prism. The effect data of the positive control 3,5-dichlorophenol (Figure 8) are in the same range as in previous studies.

The effects of the reference mixtures (Mix14 1X and 10X EQS, and Mix19 1X EQS) and the solvent control (SC) are not significantly different (ANOVA, Bonferroni, $p<0.05$), therefore all the measured effects can be attributed to the solid phase extraction (SPE). Mix19 1X EQS showed a slightly increased, however not statistically significant, toxic effect explainable by the two bactericides (sulfamethoxazole and triclosan) only present in Mix19 1X EQS.

Table 12. Inhibition of the bioluminescence in *V. fischeri* expressed as EC_{50} and EC_{10}. The relative enrichment factors (REF) refer to the reconstituted controls in Milli-Q water, with the same dilutions in the dose-response as for the mixtures.

Sample	EC_{50}	EC_{10}
Mix14 10X EQS (EQS-eq)	468.1	52.4
Mix14 1X EQS (EQS-eq)	432.3	79.9
Mix19 1X EQS (EQS-eq)	323.2	58.6

SC (REF)	411.9	85.0
SPE Blank (REF)	755.8	74.6
Negative Control	No effects measured	
3,5-dichlorophenol plate 1	3.64×10^{-5} M	3.26×10^{-5} M
3,5-dichlorophenol plate 2	3.74×10^{-5} M	3.47×10^{-5} M

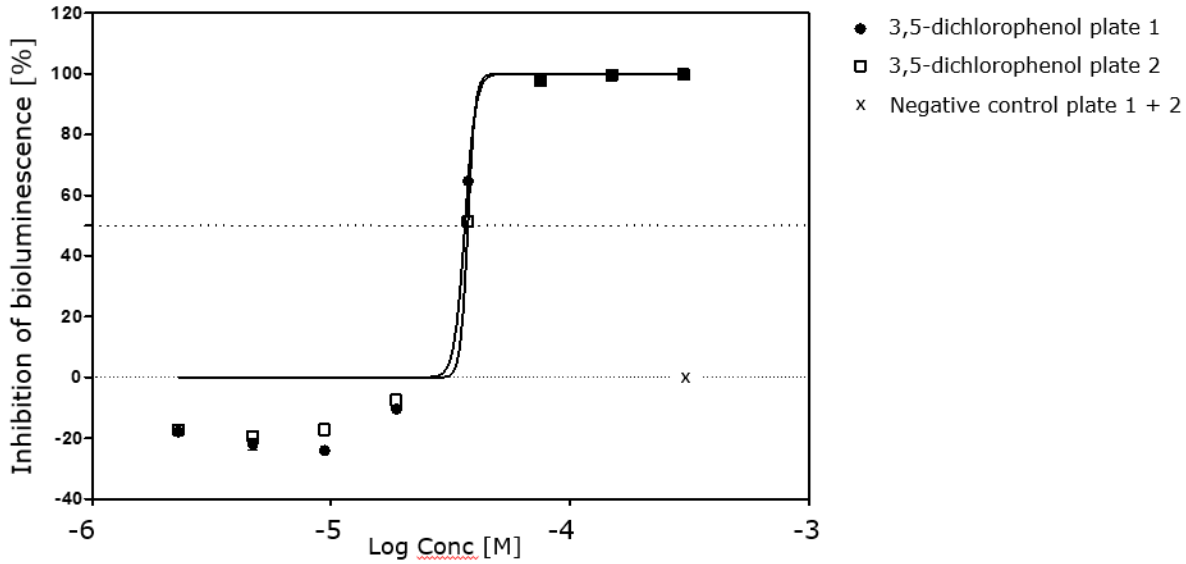


Figure 8. Inhibition of the *V. fischeri* bioluminescence in the controls. Dose-response curve of the positive control 3,5-dichlorophenol and the negative control (EtOH). Tested concentrations of 3,5-dichlorophenol range from 2.3×10^{-6} to 3.0×10^{-4} M.

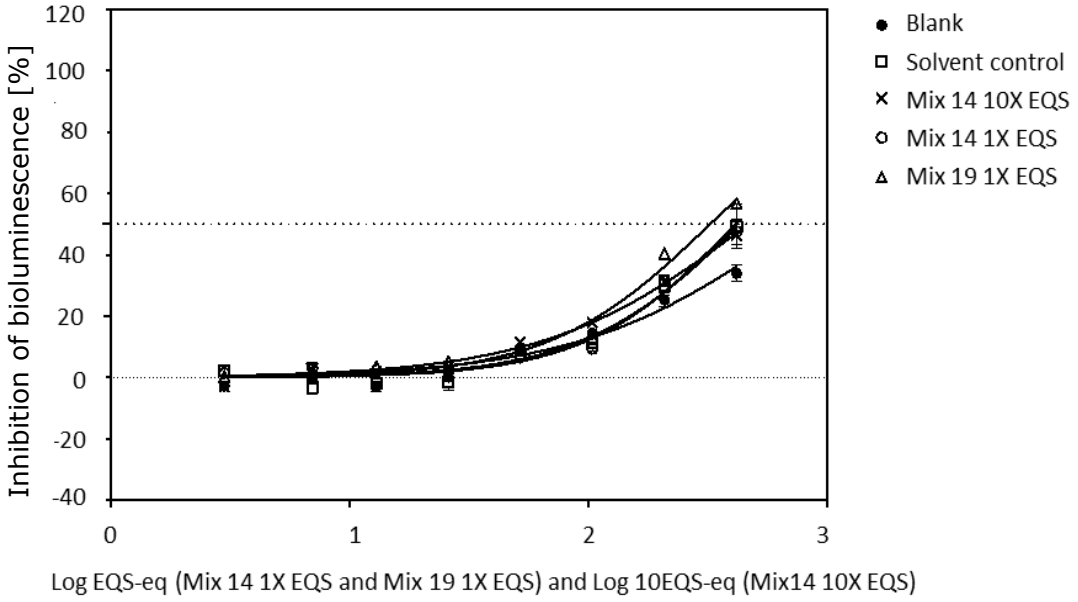


Figure 9. Inhibition of the *V. fischeri* bioluminescence in the samples. Dose-response curve of the tested reference mixtures atthe concentrations ranging from REF 3 to 417 for the reconstituted samples.

ISPRA

For exposure to the three mixtures, there was a time-dependent increase in luminescence of *V. fischeri* using the Microtox test (Table 13). Therefore, no toxicity effect was observed.

Table 13. Effect for the Microtox® test with *V. fischeri*.

Sample	Effect on luminescence with respect to the control		
	5 min	15 min	30 min
Mix14 10X EQS	3% increase	11% increase	n.d.
Mix14 1X EQS	18% increase	30% increase	40% increase
Mix19 1X EQS	16% increase	31% increase	44% increase

UNIPMN

As shown in Figure 10, no effect on *V. fischeri* bioluminescence was observed when comparing samples exposed to the reference mixtures with the control. A slight inhibition of bioluminescence was registered for Mix14 10X EQS and Mix19 1X EQS, however statistical significance was not attained.

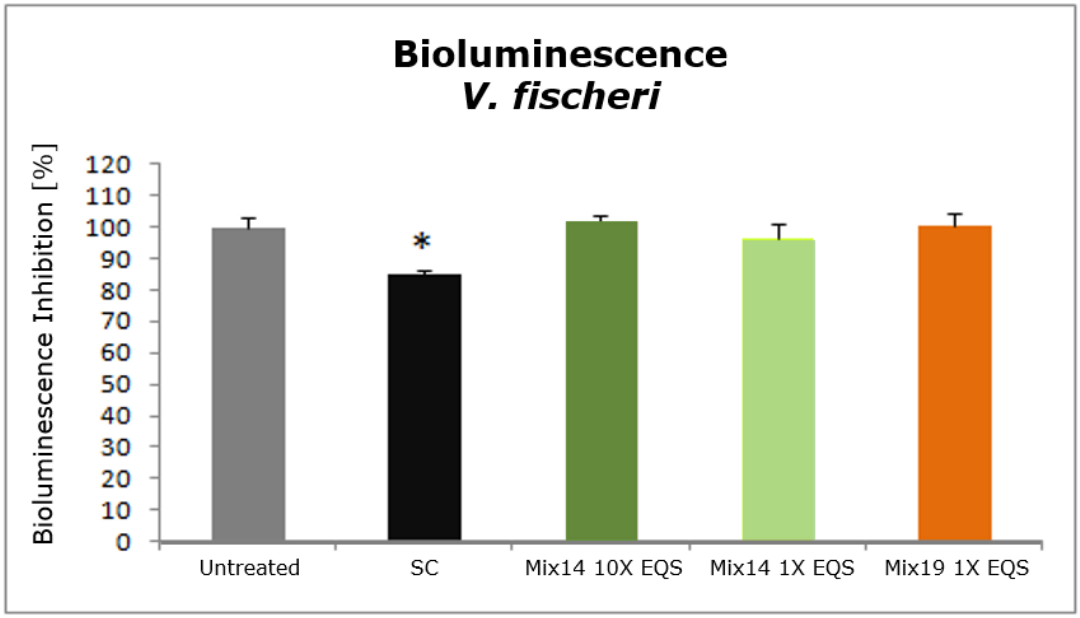


Figure 10. Inhibition of the bioluminescence in *V. fischeri*.

5.2.2 *Pseudokirchneriella subcapitata*

5.2.2.1 Inhibition of growth

RECETOX

As indicated in Table 14, Mix14 10X EQS induced a higher *P. subcapitata* growth inhibition after 96 h of exposure compared to the mixtures with less concentrated chemicals. The effect of Mix19 1X EQS was lower than that of Mix14 1X EQS suggesting an antagonist action exerted by additional substances present in Mix19.

Table 14. Effects of studied samples (1x concentration, no enrichment) for 96 h algal growth rate inhibition tests with *P. subcapitata*.

Sample	Effect compared to solvent control (growth inhibition)
Mix14 10X EQS	31%
Mix14 1X EQS	20%
Mix19 1X EQS	16%

NOTE: very low stimulation of growth in solvent control, statistically not significant

DTU

Table 15 shows a summary of results obtained in algal tests carried out at DTU. All solvents included in Mix14 10X EQS, Mix14 1X EQS and Mix19 1X EQS were assessed at a 500 times dilution corresponding to the highest test concentration in the performed tests and no statistically significant inhibition was observed.

Table 15. Effect concentrations for 72 h algal growth rate inhibition tests with *P. subcapitata*. All values are given in dilution factor relative to the originally received samples.

Sample	EC_{10} (dilution factor)	EC_{20} (dilution factor)	EC_{50} (dilution factor)
Mix14 10X EQS	<500*	<500*	<500*
Mix14 1X EQS	8000 (-**;206)	535* (146;-*)	<500
Mix19 1X EQS	<500*	<500*	<500*

* Highest tested concentration was a dilution factor of 500.
** Lowest tested concentration was a dilution factor of 8000.
- value outside of tested range/95% confidence intervals cannot be estimated
NOTE: <500 indicates that no effect was seen at the highest tested concentration

ISPRA

After 72 h of exposure to the reference mixtures, only Mix14 10X EQS induced a slight growth inhibition of *P. subcapitata*. For Mix14 1X EQS and Mix19 1X EQS, the absence of effects was contrasted by a high growth inhibition from solvents (Table 16).

Table 16. Growth rate inhibition in *P. subcapitata* after 72 h exposure.

Sample	Growth inhibition of Mix	Growth inhibition of Solvent
Mix14 10X EQS	13.7 %	3.9 %
Mix14 1X EQS	0.4%	35.9 %
Mix19 1X EQS	-	36 %

ECOTOX

Table 17 presents the data based on and derived from the nonlinear fit generated by GraphPad Prism 5 (for the detailed fit parameters see Table 17). The EC_{50} of the reference compound diuron are in the same range as in previous studies.

Figure 11A shows the measured effects on the growth rate of reference compound diuron while Figure 11B outlines the fitted dose-response curve for the exposure to Mix14 1X EQS, Mix14 10X EQS, Mix19 1X EQS, the SPE blank and the solvent control.

Table 17. Effect data of the inhibition of growth rate in *P. subcapitata*. The relative enrichment factors (REF) refer to the reconstituted controls in Milli-Q water, with the same dilutions in the dose response as for the mixtures.

Sample	EC_{50}	EC_{10}
Mix14 10X EQS (EQS-eq)	105.0	6
Mix14 1X EQS (EQS-eq)	131.90	22.51
Mix19 1X EQS (EQS-eq)	116.00	29.95
Solvent Control	No effects measured up to an REF 100	
SPE Blank	No effects measured up to an REF 100	
Diuron plate 1	1.70×10^{-7} M	2.82×10^{-8} M
Diuron plate 2	2.29×10^{-7} M	1.82×10^{-8} M

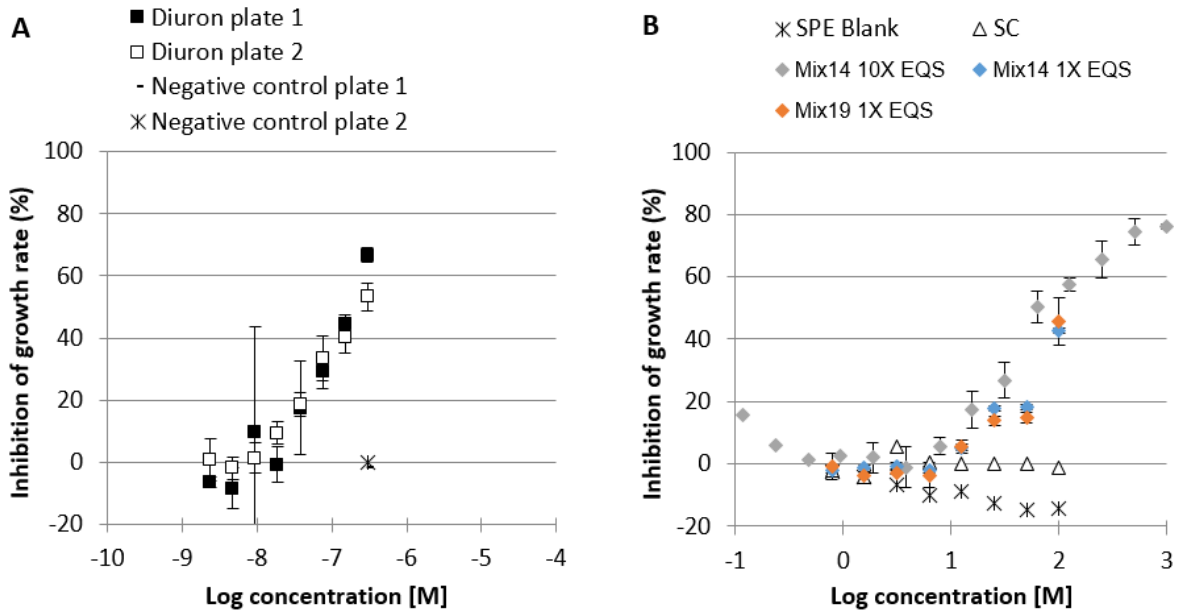


Figure 11. Inhibition of the growth rate in *P. subcapitata*. (A) Dose-response curve of the positive control diuron and the negative control. Tested concentrations of diuron range from 2.3×10^{-9} to 3.0×10^{-7} M. (B) Dose-response curve of the reference mixtures, solvent control (SC) and the SPE blank. The concentrations of the samples range from 0.03 to 1000 EQS-eq of the single chemicals in Mix14 10X EQS and from 0.8 to 100 EQS-eq for Mix14 1X EQS and Mix19 1X EQS.

UNIPMN

The three mixtures inhibited the bioluminescence in *P. subcapitata* compared to the control (Figure 12). A slightly stronger effect was observed from Mix14 10X EQS and Mix19 1X EQS than from Mix14 1X EQS. The highest inhibition of bioluminescence was induced by the solvent control.

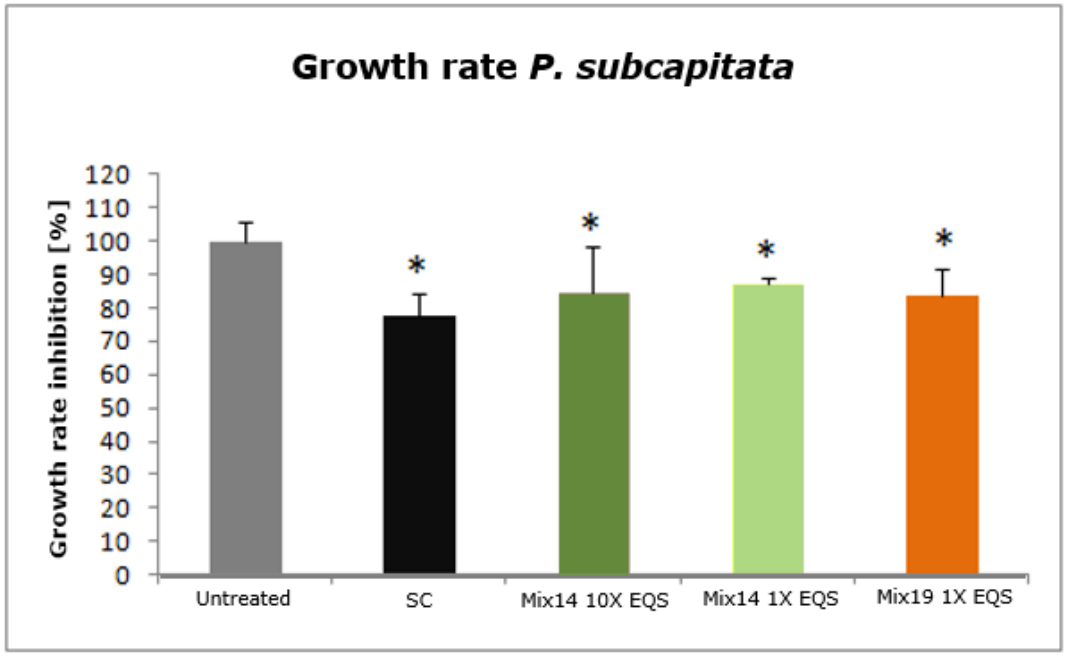


Figure 12. Inhibition of the bioluminescence in *P. subcapitata*.

5.2.2.2 Inhibition of photosynthesis

ECOTOX

Table 18 and Figure 13 present the data based on and derived from the nonlinear fit generated by GraphPad Prism 5. The EC_{50} of the reference compound diuron are in the same range as in previous studies. The presented data for the DEQ are comparable to the estimated DEQ values (see Table 19 and Figure 13) based on the relative potencies of the four herbicides diuron, atrazine, isoproturon and simazine (Table 20).

Table 18. Effect data of the inhibition of the photosynthesis in *P. subcapitata*. EC_{50}, EC_{10} and DEQ of reference mixtures, controls and diuron. The relative enrichment factors (REF) refer to the reconstituted sample in nanopure water.

Sample	EC_{50}	EC_{10}	DEQ
Mix14 10X EQS	7.3 EQS-eq	0.5 EQS-eq	5.02 µg/$L_{reconstituted\ sample}$
Mix14 1X EQS	10.95 EQS-eq	0.82 EQS-eq	0.33 µg/$L_{reconstituted\ sample}$
Mix19 1X EQS	12.56 EQS-eq	0.94 EQS-eq	0.29 µg/$L_{reconstituted\ sample}$
Solvent Control	No effects measured up to an REF 100		
SPE Blank	No effects measured up to an REF 100		
Diuron plate 1	1.73×10^{-8} M	2.10×10^{-9} M	-
Diuron plate 2	1.41×10^{-8} M	1.31×10^{-9} M	-

Table 19. Estimated DEQ of the samples Mix14 10X EQS, Mix14 1X EQS, Mix19 1X EQS and the predictability of the estimation. Data based on the relative potencies and their known concentrations of diuron, atrazine, isoproturon and simazine. The estimated DEQ of a sample is calculated by multiplying the known sample concentration by the relative potency of the substance and then adding up all calculated concentration of the four herbicides. The prediction coefficient is the quotient of the estimated and experimental DEQ.

Sample	Estimated DEQ [µg/L]	Experimental DEQ [µg/L]	Prediction coefficient
Mix14 10X EQS	3.58	5.11	0.71
Mix14 1X EQS	0.36	0.30	1.08
Mix19 1X EQS	0.36	0.26	1.23

Table 20. Relative potencies of three herbicides, compared to the reference diuron, for the 2 h endpoint in the IPAM. The relative potency of a substance was calculated by dividing the EC_{50} of the substance by the EC_{50} of the reference diuron (determined based on experimental studies at the Ecotox Centre, unpublished).

Name	Relative potency
Diuron	1
Atrazine	0.0797
Isoproturon	0.1465
Simazine	0.0662

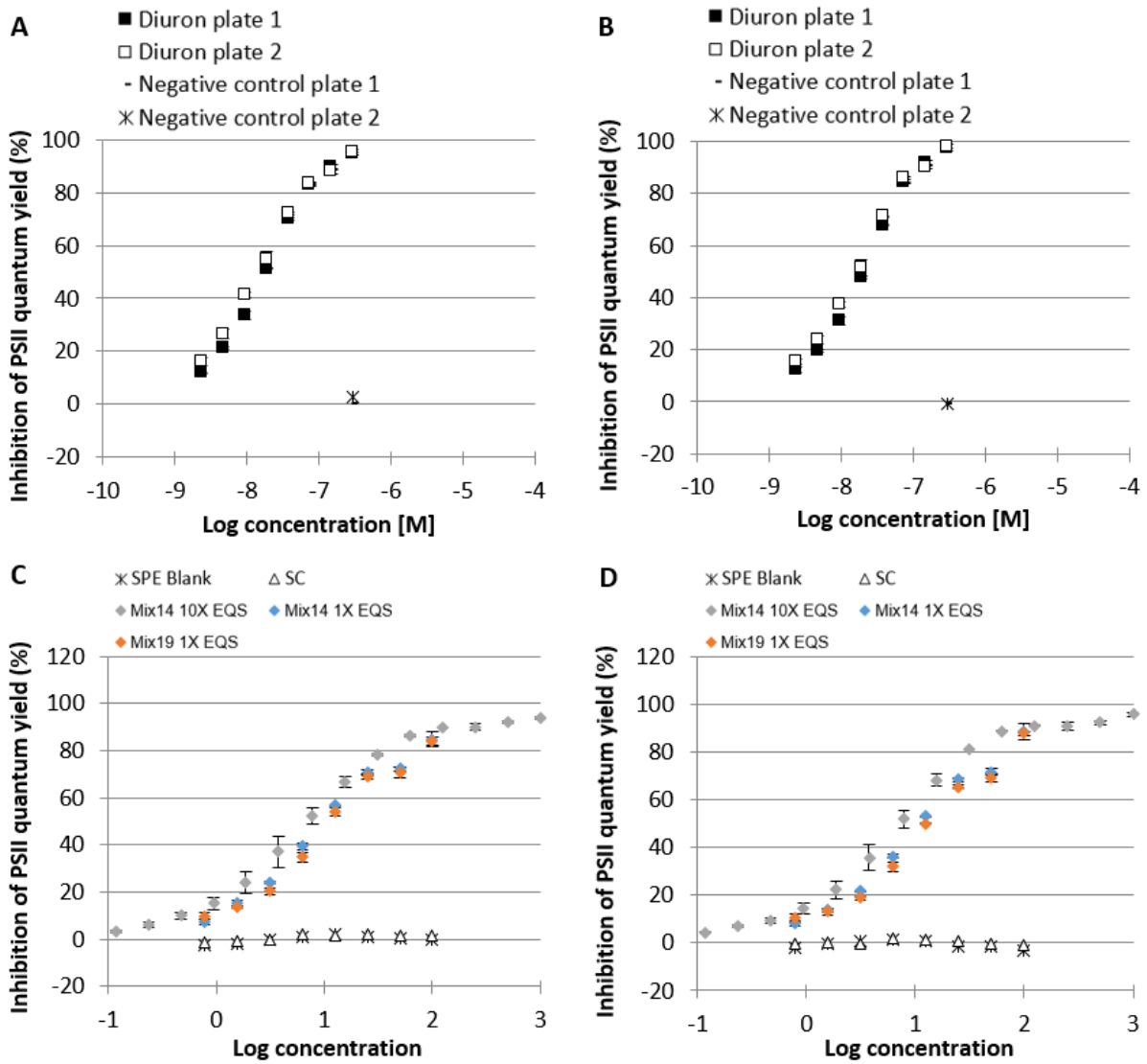


Figure 13. Inhibition of photosynthesis in *P. subcapitata*. Inhibition of the photosystem II. Dose-response curve of the positive control diuron and the tested negative control for 2 h (A) and 24 h (C) exposure. Tested concentrations of diuron range from 2.3×10^{-9} to 3.0×10^{-7} M. Dose-response curve of the samples Mix14 10X EQS, Mix14 1X EQS, Mix19 1X EQS, solvent control (SC) and the SPE blank for 2 h (B) and 24 h (D) exposure. The concentration of the samples ranges from 0.03 to 1000 EQS-eq of the single chemicals in Mix14 10X EQS, and 0.8-100 EQS-eq for Mix14 1X EQS and Mix19 1X EQS.

5.2.3 *Chlamydomonas reinhardtii*

EAWAG

Effects on photosynthesis

The exposure to the mixture produced a clear dose-dependent inhibition of growth (Figure 14) and photosynthetic yield in *C. reinhardtii*. Figure 15 shows the inhibition of photosynthesis at concentrations ranging from 0.2x to 100x EQS with similar trendsobserved after 2 and 24 h. No inhibition was observed with the methanol solvent. The algae showed a clear dose-dependent response at both growth and photosynthetic yield. From the dose-response curve, EC_{10} and EC_{50} values for photosynthesis at 2 h and

24 h, and growth at 24 h were determined (Table 21) and the estimates will be used for further exposure studies with the Mix14 and Mix19.

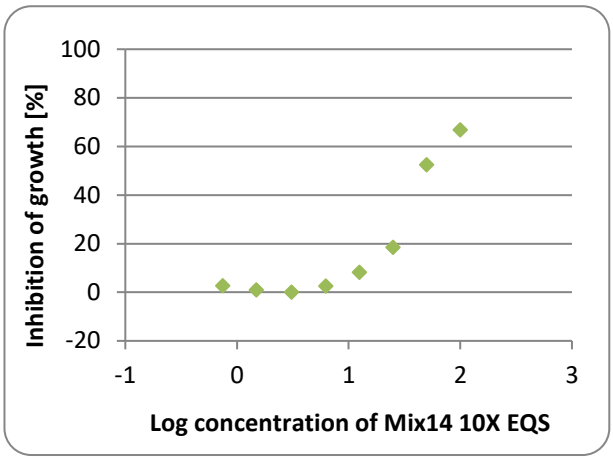


Figure 14. Inhibition of the *C. reinhardtii* growth after 24 h exposure. Dose response curve of the reaction Mix14 10X EQS. The concentration of the samples ranges from 0.2x to 100x EQS.

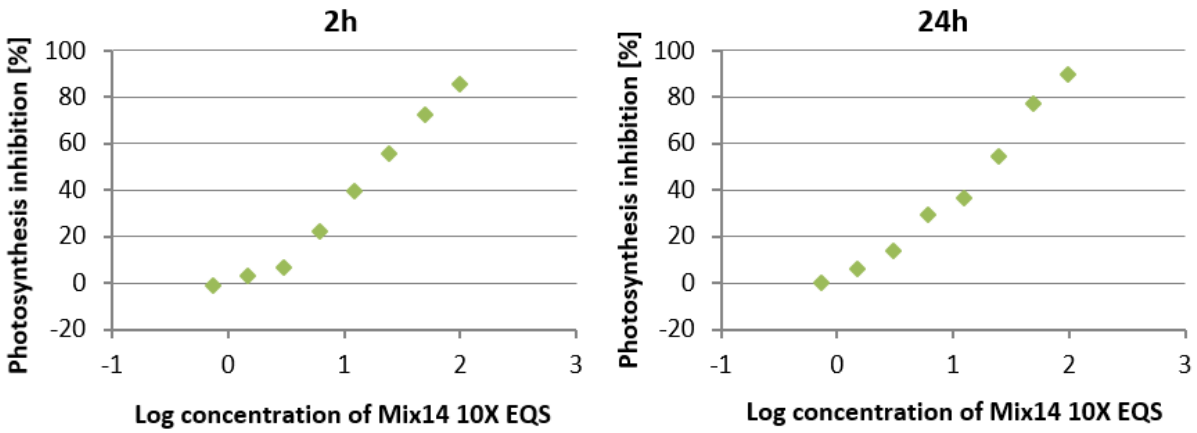


Figure 15. Inhibition of the photosystem II in *C. reinhardtii* after 2 and 24 h exposure. Dose response curve of the reaction Mix14 10X EQS. The concentration of the samples ranges from 0.2x to 100x EQS.

Table 21. The effect concentration (EC) values for physiological endpoints of photosynthetic yield (PS) in *C. reinhardtii* at 2 and 24 h, and growth at 24 h. The values represent the concentration of the mixture in EQS-eq.

EC	PS 2h	PS 24h	Growth
EC_1	0.6	1.1	1.9
EC_{10}	3.5	5.0	8.8
EC_{50}	17.9	19.9	36.0

5.2.4 *Thalassiosira pseudonana*

JRC

Figure 16 shows a growth inhibition of *T. pseudonana* induced by the reference mixtures at different concentrations and measured at three time points of exposure. The effects were significant for concentrations equivalent to 5-fold EQS and already visible at 2-fold EQS. In all cases, growth inhibition was more pronounced after 24 h of culture.

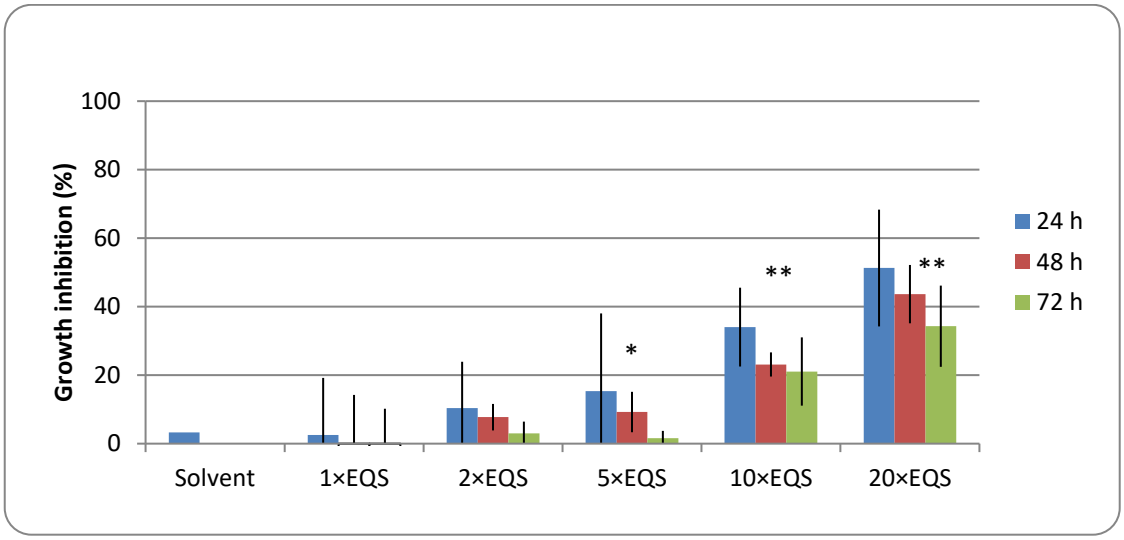


Figure 16. Effect of the reference mixtures on the growth of the diatom *Thalassiosira pseudonana*. A dose-response was tested with final concentrations in the culture media ranging from the lowest (1×EQS) to the highest containing each chemical at the concentration corresponding to 20×EQS. The effect of the solvent control was also assessed. The growth rate of the cultures was measured at 24, 48 and 72 h. Asterisks indicate significant growth inhibition with respect to the solvent control for 48 h exposure (paired *t*-test) with *p<0.05 and **p<0.01.

5.2.5 *Daphnia magna*

5.2.5.1 Daphnia magna acute immobilisation test

RECETOX

Almost complete immobilisation of *D. magna* was observed upon exposure to Mix 10X EQS, while no effects were induced after 48 h by the mixtures with lower concentrations of chemicals (Table 22).

Table 22. Effect of mixtures (1x concentration, no enrichment) on the acute immobilisation test with *D. magna*, for a period of exposure of 48 h.

Sample	Immobilisation
Mix14 10X EQS	90%
Mix14 1X EQS	no effect
Mix19 1X EQS	no effect

DTU

Tables 23 and 24 show a summary of results obtained in the crustacean tests carried out at DTU. All solvents included in RM040-042 were assessed at a 500 times dilution corresponding to the highest test concentration and no statistically significant inhibition was observed.

Table 23. Effect concentrations for 24 h immobilisation tests with *D. magna*. All values are given in dilution factor relative to the originally received samples.

Sample	EC_{10} (dilution factor)	EC_{20} (dilution factor)	EC_{50} (dilution factor)
Mix14 10X EQS	3.3 EQS-eq	4.4 EQS-eq	8.0 EQS-eq
Mix14 1X EQS	1.1 EQS-eq	>2 EQS-eq*	>2 EQS-eq*
Mix19 1X EQS	>2 EQS-eq*	>2 EQS-eq*	>2 EQS-eq*

* Highest tested concentration was a dilution factor of 500 (corresponding to 2 EQS-eq)

Table 24. Effect concentrations for 48 h immobilisation tests with *D. magna*. All values are given in dilution factor relative to the originally received samples.

Sample	EC_{10} (dilution factor)	EC_{20} (dilution factor)	EC_{50} (dilution factor)
Mix14 10X EQS	1.2 EQS-eq	1.6 EQS-eq	2.8 EQS-eq
Mix14 1X EQS	<0.125 EQS-eq**	0.45 EQS-eq	>2 EQS-eq*
Mix19 1X EQS	<0.125 EQS-eq**	1.03 EQS-eq	>2 EQS-eq*

* Highest tested concentration was a dilution factor of 500 (corresponding to 2 EQS-eq)
** Lowest tested concentration was a dilution factor of 8000 (corresponding to 0.125 EQS-eq)
NOTE <500 indicates that no effect was seen at the highest tested concentration; >8000 indicates that the EC10 value was extrapolated to be outside of the range of concentrations used

ISPRA

Effect concentrations for 48 h immobilisation tests with *D. magna*.

Table 25 shows a summary of results obtained in the crustacean tests carried out at ISPRA. Effects induced by Mix14 10X EQS reached 70% of *D. magna* immobilisation. No significant effects were observed from the mixtures containing lower concentrations of substances.

Table 25. Immobilisation of *D. magna* in 24 h tests.

Sample	% effect 24 h		
	Replicate 1	Replicate 2	Average
Negative Control	0	5	2.5
Solvent Control	0	5	2.5
Mix14 10X EQS	70	60	65
Mix14 1X EQS	5	20	12.5
Mix19 1X EQS	5	0	2.5

5.2.5.2 Dapnia magna reproduction test

RECETOX

Effects of Mix14 1X EQS and Mix19 1X EQS were negligible with respect to the solvent control (Table 26), while Mix14 10X EQS caused mortality before the reproduction could occur. The observed inhibition in *D. magna* reproduction with the two mixtures might be caused mainly by methanol as solvent.

Table 26. Effect of mixtures (1x concentration, no enrichment) on the reproduction test with *D. magna* after a period of exposure of 21 days.

Sample	Inhibition of reproduction[a]
Mix14 10X EQS	100% mortality after 3 days, no reproduction could be evaluated
Mix14 1X EQS	31±37% (ns)
Mix19 1X EQS	23±24% (ns)

[a] The solvent control induced a 62% inhibition of reproduction compared to media control
ns: statistically not significant

5.2.6 *Dictyostelium discoideum*

UNIPMN

As shown in Figure 17, there was no effect of Mix14 10X EQS, Mix14 1X EQS and Mix19 1X EQS on mortality of *D. discoideum*. Additionally, there was a decreased reproduction rate for the three mixtures although the observed effect could be explained by the solvent exposure. Moreover, decreased lysosomal membrane stability was registered for Mix14 10X EQS > Mix19 1X EQS > solvent control, while no effect of Mix14 1X EQS was observed (Figure 17).

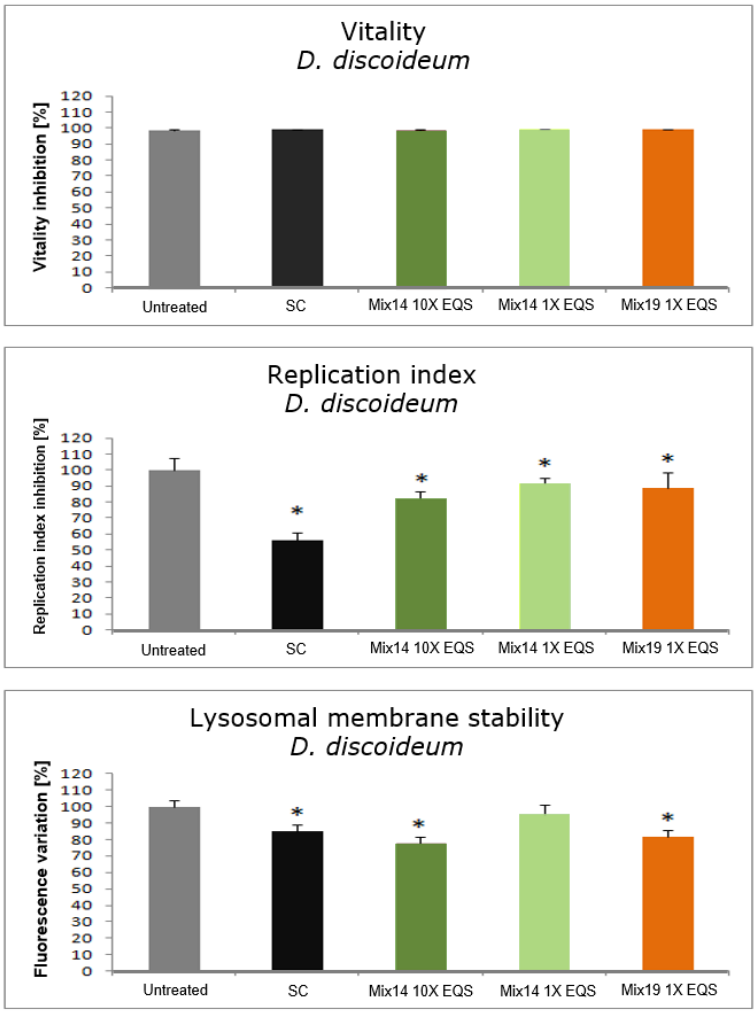


Figure 17. Effects of the reference mixtures on mortality, replication and lysosomal membrane stability of *D. discoideum*.

5.2.7 MTT test

5.2.7.1 RTG-2 cells

ISPRA

An effect was observed for Mix14 10X EQS with a decreased survival rate of RTG-2 cells, while only a slight effect was observed for Mix14 1X EQS and Mix19 1X EQS with respect to the solvent control (Table 27).

Table 27. Effect of mixtures on the MTT test with RTG-2 cells.

Sample	Survival rate
Mix14 10X EQS	82%
Mix14 1X EQS	90%
Mix19 1X EQS	94%
Solvent Control	95%

5.2.7.2 Primary rainbow trout gill cells

DNSC

No significant effect on primary rainbow trout (*Oncorhynchus mykiss*) cell viability was observed after 20 h of treatment to reaction mixture Mix14 10X EQS compared to the solvent control. The reference mixture caused an average loss of cell viability of 10% (Figure 18).

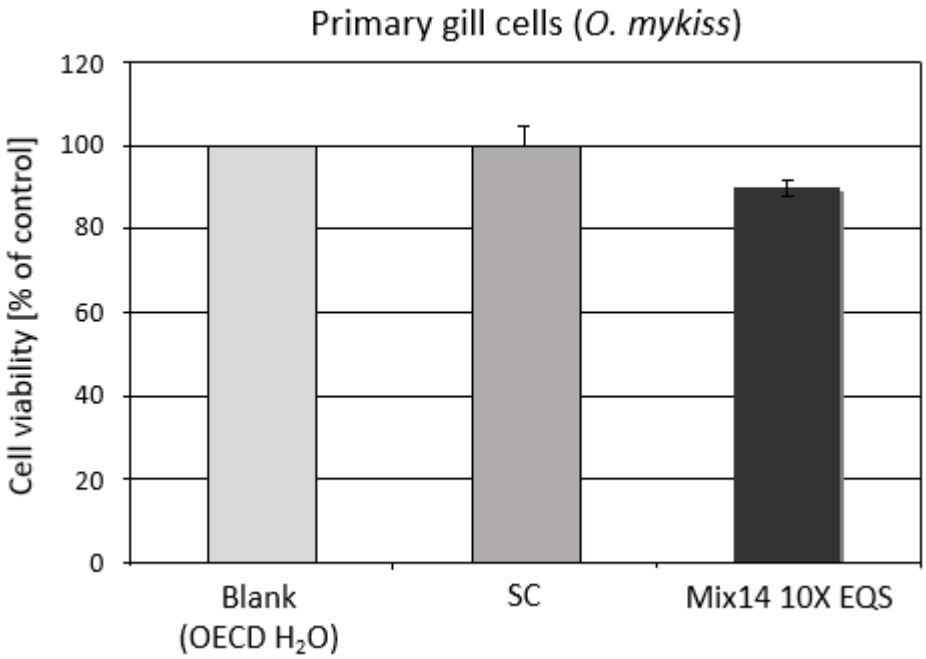


Figure 18. Viability of primary gill cells in rainbow trout upon 20 h exposure to Mix14 10X EQS. Results are expressed as percent of live cells with reference to the solvent control (SC).

5.2.7.3 Other cell lines

Four additional human cell lines derived from kidney tissue (RPTEC/TERT1), umbilical cord (HUVEC/TERT), liver tissue (HepG2) and breast carcinoma (MCF7) were treated with the exposure Mix14 10X EQS (1:500, 1:1000, 1:2000), Mix14 1X EQS (1:500, 1:1000) and Mix19 1X EQS (1:1000) for 24 h. Thereafter, the plates were analysed using MTT assays. No cytotoxic effects could be monitored after treating HepG2, MCF7 and RPTEC/TERT1 cells. In contrast, HUVEC/TERT7 cells treated with the solvent (methanol, nitric acid; 1:500 dilution, highest concentration of solvent in Mix14 10X EQS, Mix14 1X EQS) responded significantly compared to cells treated with exposure mixture Mix14 10X EQS, Mix14 1X EQS and Mix19 1X EQS at different dilutions, where higher MTT signals were observed (Figure 19).

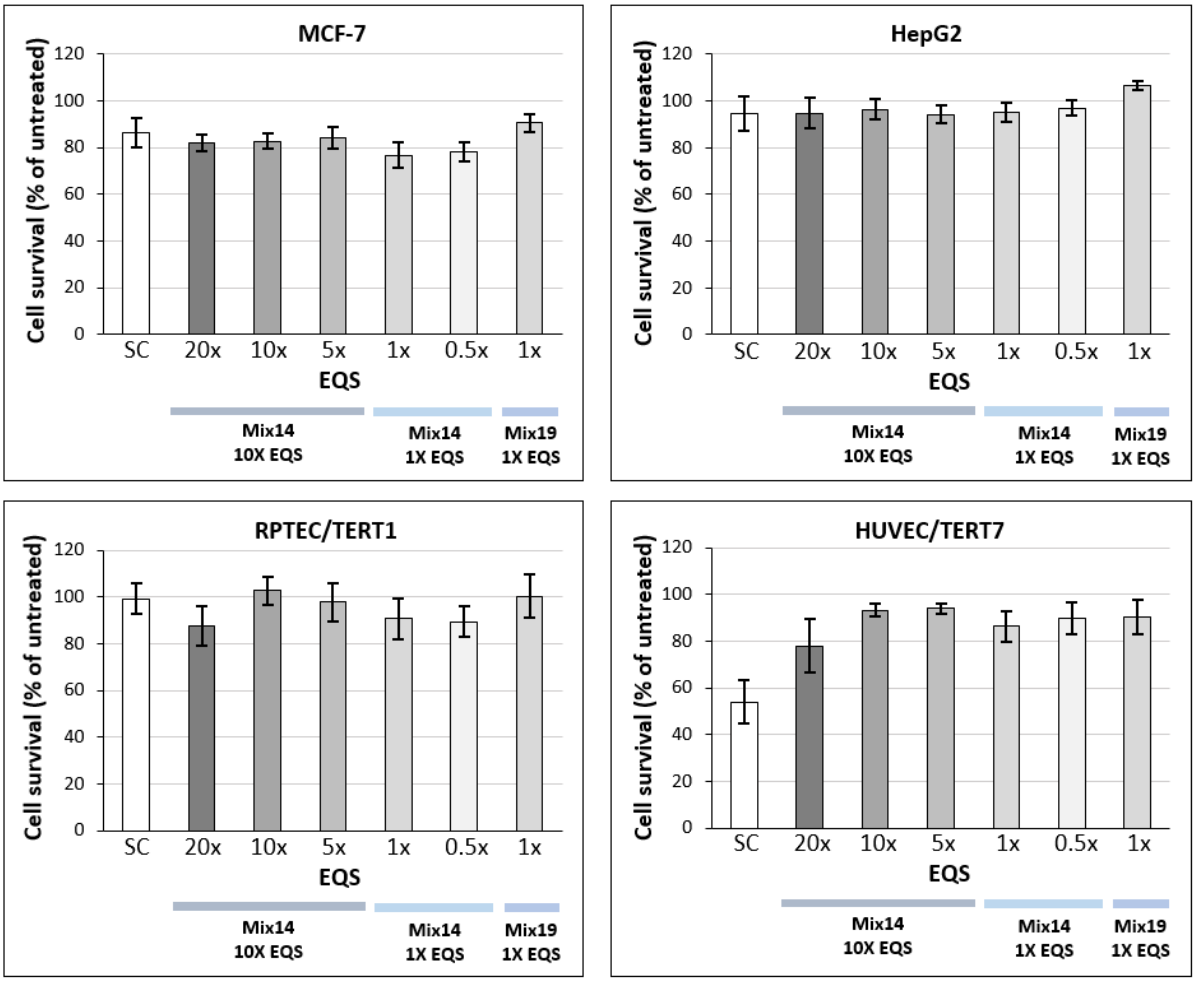


Figure 19. Mixture effects on the survival or proliferation of different cell lines using the MTT test. The cell survival rate was calculated with respect to untreated cultures. Different concentrations of Mix14 10X EQS and Mix14 1X EQS were tested and displayed in the graphs as fold concentration with respect to EQS values of single compounds in the mixtures, while Mix19 1X EQS was tested as 1-fold EQS equivalent concentration only. Error bars represent the standard deviation of the mean for six different measurements.

5.2.8 Nuclear red test

RECETOX

No effect was observed on the cytotoxicity of H4IIE-luc cells using the nuclear red test, for any of the mixtures when compared to solvent control.

5.2.9 Primary cultures of hepatocytes from juvenile Atlantic salmon

NIFES

None of the applied mixtures induced cytotoxic effects in exposed primary hepatocytes as measured with the xCELLigence cytotoxicity system (Figure 20).

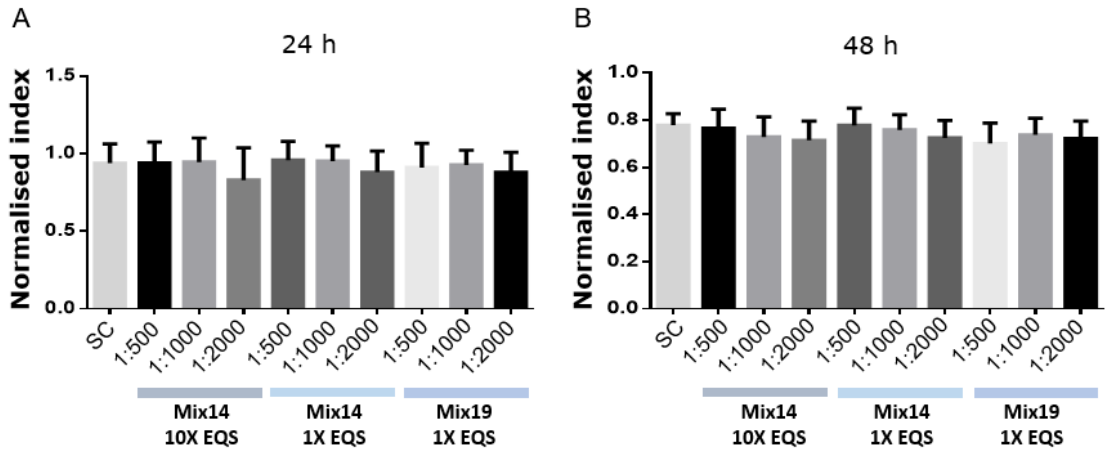


Figure 20. Response curves for normalised cell index values obtained for primary Atlantic salmon hepatocytes. The cell replicates (n = 4) were exposed to Mix14 10X EQS, Mix14 1X EQS and Mix19 1X EQS. The values represent the mean±SE of four replicates. The analyses showed no significant difference between the control and the exposed groups ($p<0.05$).

5.3 Effects on embryo toxicity and development

5.3.1 Fish Embryo Toxicity (FET) test with zebrafish *(Danio rerio)*

RECETOX

Parameters monitored were total mortality, number of hatched embryos, number of defected embryos, deformities (head, tail deformities, absence of gas bladder), underdeveloped embryos and length. After exposure for 120 h, effects in several endpoints were observed for Mix14 10X EQS and Mix19 1X EQS, but not for Mix14 1X EQS, as can be seen in Table 28. On shorter times of exposure, only Mix14 10X EQS triggered significant effects, particularly in terms of number of defected embryos after 72 h and in number of hatched embryos after 96 h (representative images are shown in Figure 21).

Table 28. Effect of mixtures on the fish embryo toxicity test (FET) with zebrafish with 120 h exposure to 1x equivalent concentration (no enrichment applied).

Sample	Embryo toxicity effects
Mix14 10X EQS	Effects observed in number of defected embryos – absence of gas bladder, head deformities and underdeveloped embryos were observed the most often
Mix14 1X EQS	No significant effects observed
Mix19 1X EQS	Effects observed in number of defected embryos, number of underdeveloped embryos and length

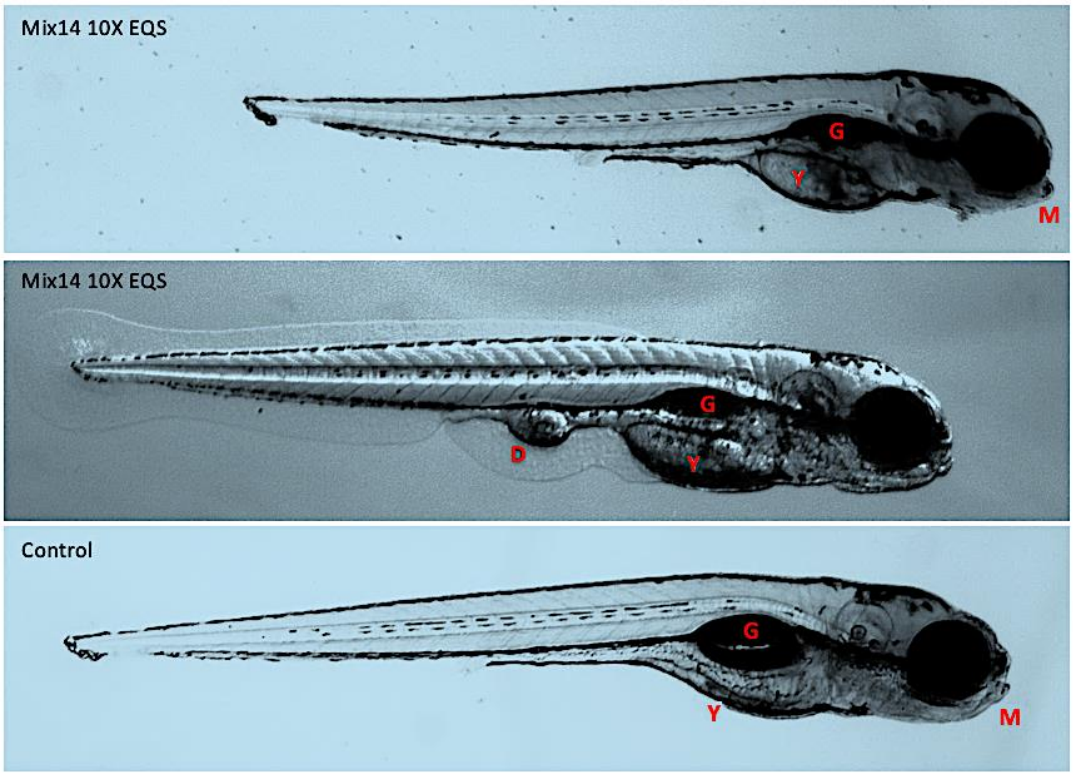


Figure 21. Representative endpoints observed at 120 hpf (exposures to 1x equivalent concentrations) using the FET bioassay. Two embryos exposed to Mix14 10X EQS solution are shown on the top panel, and a control embryo is shown below. The top embryo is an example of a typically underdeveloped embryo in this experiment. The gas (swimming) bladder (G) is not inflated, the head is deformed which is especially visible at mouth region (M), yolk is not fully consumed (Y) and embryo is shorter. The second exposed embryo (middle panel) has also deformation (D) nearby the anus region, not inflated gas (swimming) bladder (G) and not fully consumed yolk (Y).

5.3.2 Frog Embryo Teratogenesis Assay (FETAX)

RECETOX

No effect on embryo length was observed for neither of the mixtures nor the solvent control. However, as can be seen in Table 29, a significant amount of malformations was observed for the embryos exposed to the mixtures, when compared to the solvent control, and the number of malformations increased with increased concentration of the chemicals (Mix14 10X EQS).

Table 29. Effect of mixtures in the frog embryo teratogenicity assay (FETAX) with 96 h exposure - all samples tested at 1x equivalent concentration.

Sample	Embryo toxicity effects
Mix14 10X EQS	62±10% malformed embryos
Mix14 1X EQS	43±12% malformed embryos
Mix19 1X EQS	34±14% malformed embryos
Solvent Control[a]	15±12% malformed embryos

[a] DMSO was used as solvent control because of the toxicity of methanol to frog embryos

5.4 Estrogenicity

5.4.1 YES (yeast screen assay)

ECOTOX

Table 30 presents the effect data based on and derived from the nonlinear fit generated by GraphPad Prism 5 (for the detailed fit parameters see Table 32). The EC_{50} values of the reference compound 17β-estradiol are in the same range as in previous studies. Concerning the fact that only three substances with estrogenic activity were included (17β-estradiol, 4-nonylphenol and bisphenol A; relative potencies in Table 31), the presented EEQ data are comparable to the estimated EEQ values (1).

Table 30. Effect data of the YES assay. EC_{50}, EC_{10} and EEQ of Mix14 10X EQS, Mix14 1X EQS, Mix19 1X EQS, controls and 17β-estradiol. The relative enrichment factors (REF) refer to the reconstituted sample in nanopure water.

Sample	EC_{50}	EC_{10}	EEQ±σ
Mix14 10X EQS	89 EQS-eq	28 EQS-eq	7.12 ±0.51 ng/$L_{reconst.\ sample}$
Mix14 1X EQS	92.3 EQS-eq	18.8 EQS-eq	0.47 ±0.08 ng/$L_{reconst.\ sample}$
Mix19 1X EQS	90.5 EQS-eq	20.2 EQS-eq	0.52 ±0.09 ng/$L_{reconst.\ sample}$
Solvent Control	No effects measured up to an REF 100		
SPE Blank	No effects measured up to an REF 100		
Negative Control (EtOH)	No effects measured		
17β-Estradiol plate 1	2.29×10^{-10} M	8.96×10^{-11} M	-
17β-Estradiol plate 2	2.42×10^{-10} M	8.60×10^{-11} M	-
17β-Estradiol plate 3	2.14×10^{-10} M	7.49×10^{-11} M	-

Table 31. Relative potencies of two compounds of the reconstituted samples compared to the reference 17β-estradiol in the YES assay. (Rutishauser et al. 2004).

Name	Relative potency
17β-Estradiol	1
4-Nonylphenol	0.000025
Bisphenol A	0.00011

Table 32. Estimated EEQ of the samples Mix14 10X EQS, Mix14 1X EQS and Mix19 1X EQS and the predictability of the estimation for the YES assay. Based on the relative potencies and the known concentration of 17β-estradiol, 4-nonylphenol and bisphenol A. The estimated EEQ of a sample iscalculated by multiplying the known sample concentration by the relative potency of the substance and then adding up all calculated concentrations. The prediction coefficient is the quotient of the estimated and the experimental EEQ.

Sample	Estimated EEQ [ng/L]	Experimental EEQ [ng/L]	Prediction coefficient
Mix14 10X EQS	4.08	7.12±0.51	0.57
Mix14 1X EQS	0.41	0.47±0.08	0.87
Mix19 1X EQS	0.57	0.52±0.09	1.10

Figure 22 presents the measured effects in a dose-response curve of the reference compound 17β-estradiol. The effects of the exposure mixtures, the SPE blank and the solvent control are shown in Figure 23.

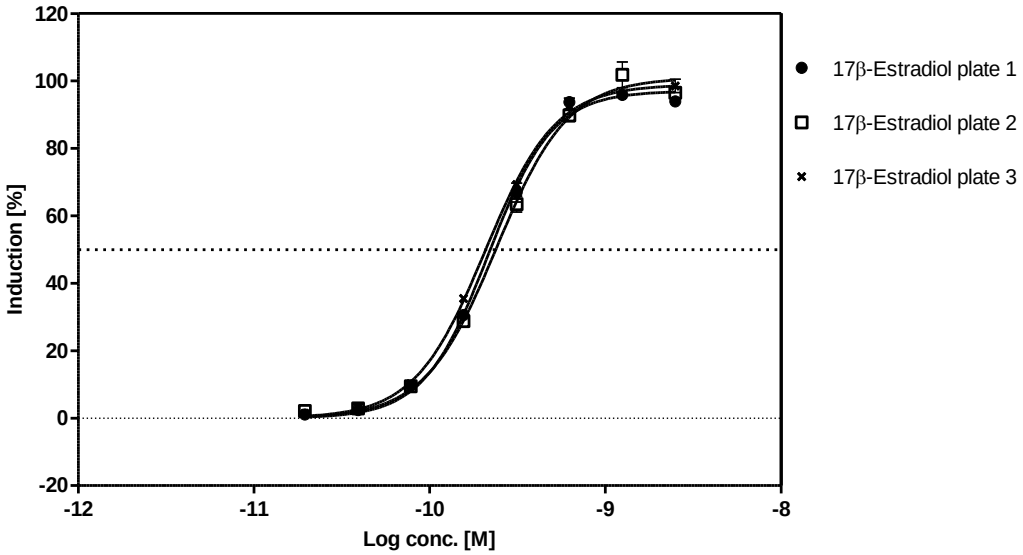


Figure 22. Induction data of the YES, reference compound. Dose-response curve of the standard 17β-estradiol, tested concentrations range from 2.50×10^{-9} to 1.95×10^{-11} M.

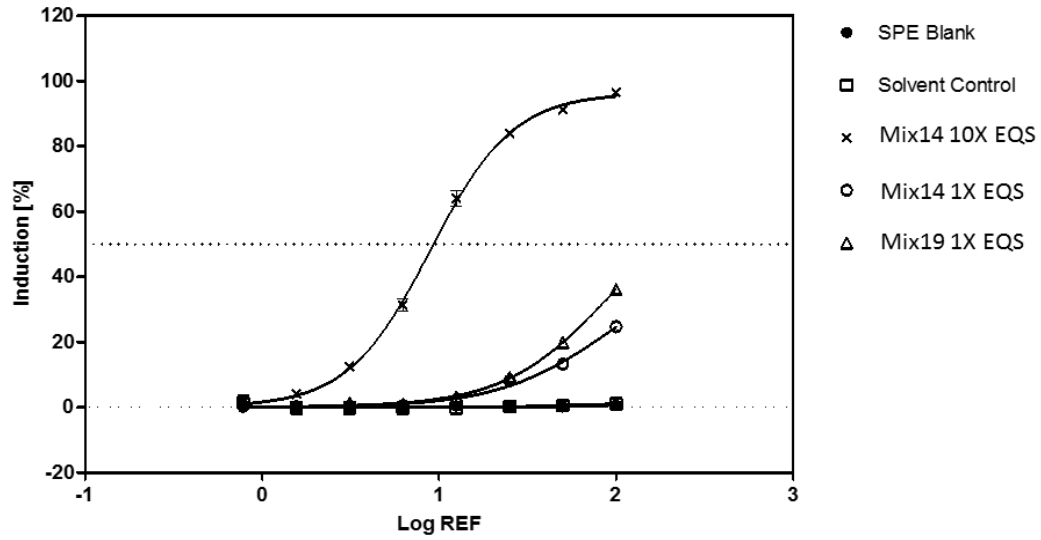


Figure 23. Induction data of the YES, samples. Dose-response curve of Mix14 10X EQS, Mix14 1X EQS and Mix19 1X EQS, xolvent control and the SPE blank. The concentration of the samples range from REF 0.78 to 100 of the reconstituted samples.

5.4.2 Estrogen receptor (ER)-activated luciferase induction

5.4.2.1 ER-CALUX®

ECOTOX

Table 33 shows the EC_{50} values and estradiol equivalent concentrations of the analysed samples. The EC_{50} values of the reference compound 17β-estradiol (Figure 24) were in the same range as in other studies (Kunz et al. 2017).

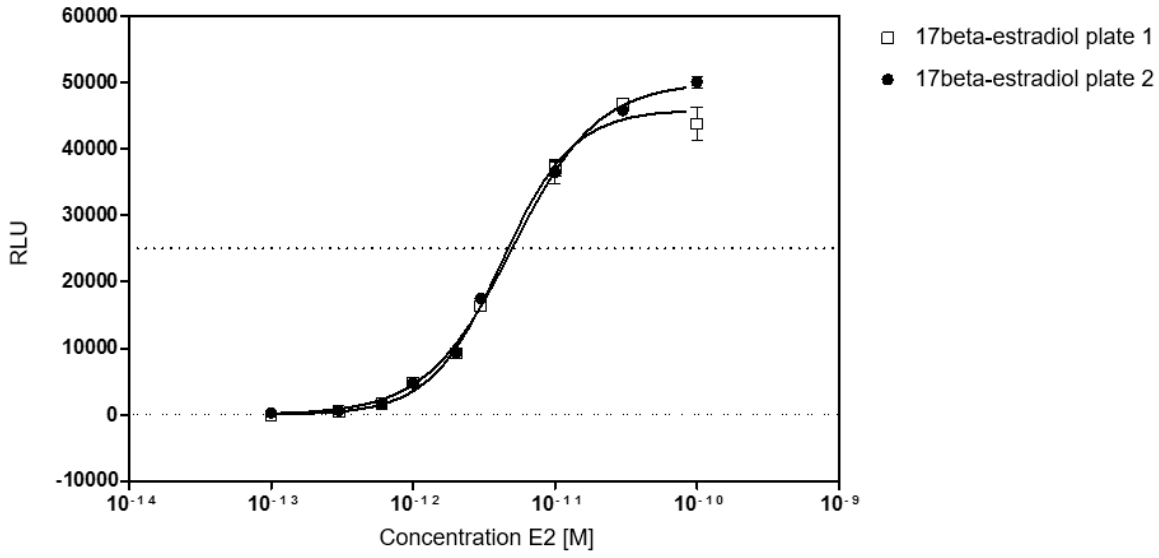


Figure 24. Dose-response curve of the standard 17β-estradiol. Tested concentrations range from 1.0×10^{-10} to 1.0×10^{-13} M.

A low effect was measured at REF 100 in the solvent control as well as in the SPE blank (Figure 25). The effect was smaller than the limit of detection, therefore it could not be

quantified. For the quantification of the samples, a REF of 33.3 or lower was used. At these REF, neither in the solvent control nor in the SPE blank effects were measured.

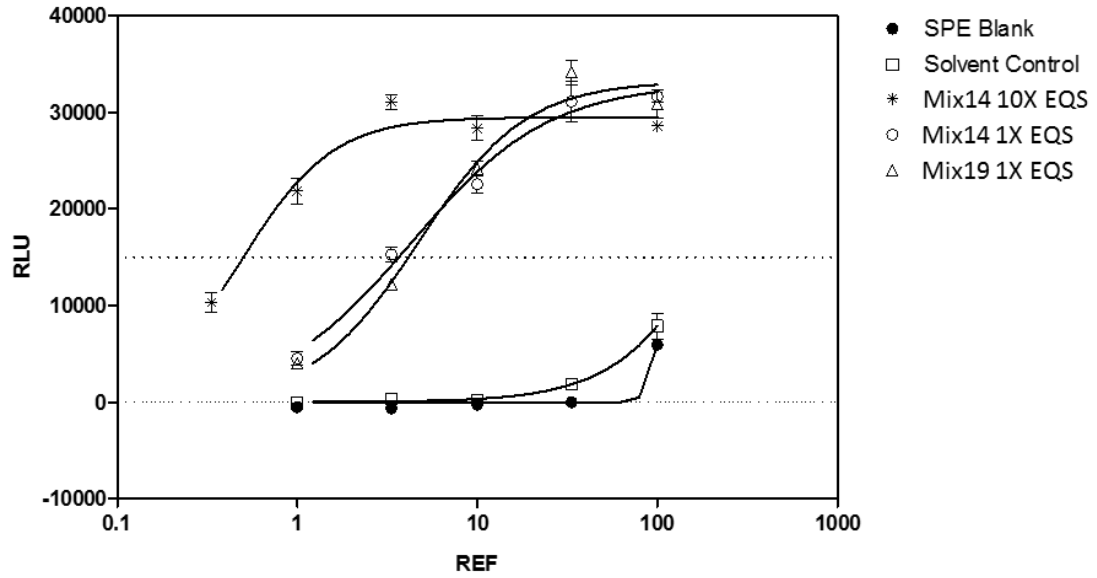


Figure 25. Estrogenic activities in the ER-CALUX. Dose-response curve of Mix14 10X EQS, Mix14 1X EQS and Mix19 1X EQS, solvent control and the SPE blank. The concentration of the samples range from REF 0.33 to 100 of the reconstituted samples.

All tested mixtures showed estrogenic activities in the ER-CALUX. Mix14 10X EQS displayed a full dose-response curve and was, as expected, the most potent one. Mix14 1X EQS and Mix19 1X EQS displayed submaximal dose-response curves and showed comparable estrogenic potencies (Figure 25).

Table 33. EC_{50} and EEQ of Mix14 10X EQS, Mix14 1X EQS and Mix19 1X EQS, solvent control, SPE blank and 17β-estradiol in the ER-CALUX. The relative enrichment factors (REF) refer to the reconstituted sample in nanopure water.

Sample	EC_{50}	EEQ±σ
Mix14 10X EQS	4.92 EQS-eq	1.1* ng/$L_{reconst.\ sample}$
Mix14 1X EQS	4.303 EQS-eq	0.18±0.08 ng/$L_{reconst.\ sample}$
Mix19 1X EQS	4.742 EQS-eq	0.10±0.04 ng/$L_{reconst.\ sample}$
Solvent Control	Low effect measured at REF 100: ~ 404.9	<LOD
SPE Blank	Low effect measured at REF 100: ~ 87.96	<LOD
17β-Estradiol plate 1	4.21×10^{-12} M	-
17β-Estradiol plate 2	4.99×10^{-12} M	-

* No standard deviation because only one concentration was used for the EEQ calculation

Estimated EEQ values were calculated for the analysed mixtures (Table 35), based on the potencies of known estrogenic substances in the ER-CALUX (Table 34). This was only

possible for three substances of the mixtures: 17β-estradiol, 4-nonylphenol and bisphenol A. For all other mixture components with reported endocrine activities, data for similar calculations were not available. The estimated EEQ values (Table 35) were about 3 to 6 times higher than the measured EEQs of the mixtures.

Table 34. Relative potencies of two estrogenic compounds of the reconstituted samples in the ER-CALUX. Compared to the reference 17β-estradiol (Sonneveld et al. 2005).

Name	Relative potency
17β-Estradiol	1
4-nonylphenol	5.9×10^{-4}
Bisphenol A	2.7×10^{-5}

Table 35. Estimated EEQ of the samples Mix14 10X EQS, Mix14 1X EQS and Mix19 1X EQS and the predictability of the estimation for the ER-CALUX. Based on the relative potencies and the known concentration of 17β-estradiol, 4-nonylphenol and bisphenol A. The estimated EEQ of a sample was calculated by multiplying the known sample concentration by the relative potency of the substance and then adding up all calculated concentrations. The prediction coefficient is the quotient of the estimated and the experimental EEQ.

Sample	Estimated EEQ [ng/L]	Experimental EEQ [ng/L] ± σ	Prediction coefficient
Mix14 10X EQS	5.77	1.1*	5.25
Mix14 1X EQS	0.58	0.18±0.08	3.22
Mix19 1X EQS	0.62	0.10±0.04	6.2

* No standard deviation because only one concentration was used for the EEQ calculation

5.4.2.2 MELN cells

ECOTOX

Figures 26 and 27 present dose-dependent induction of luciferase in MELN cells by the reference chemical estradiol and the reference mixtures Mix14 10X EQS, Mix14 1X EQS and Mix19 1X EQS. The Table 36 shows their corresponding EC_{50} and EC_{10}.

All the tested mixtures were active in the MELN assay. On the basis of EQS equivalent, Mix14 10X EQS and Mix14 1X EQS exerted the same estrogenic potency, which is logical due to the identical chemical composition. It is noteworthy that Mix19 1X EQS was significantly more active than the two other mixtures. The lowest effect (EC_{10}) was observed at the EQS level for Mix19 1X EQS and at 2.5xEQS for Mix14 10X EQS and Mix14 1X EQS.

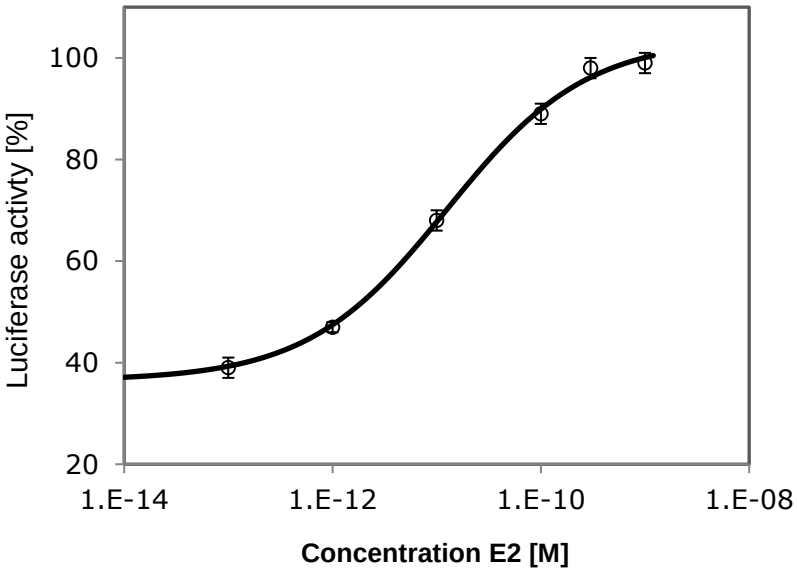


Figure 26. Induction of luciferase activity by 17β-estradiol in MELN cells.

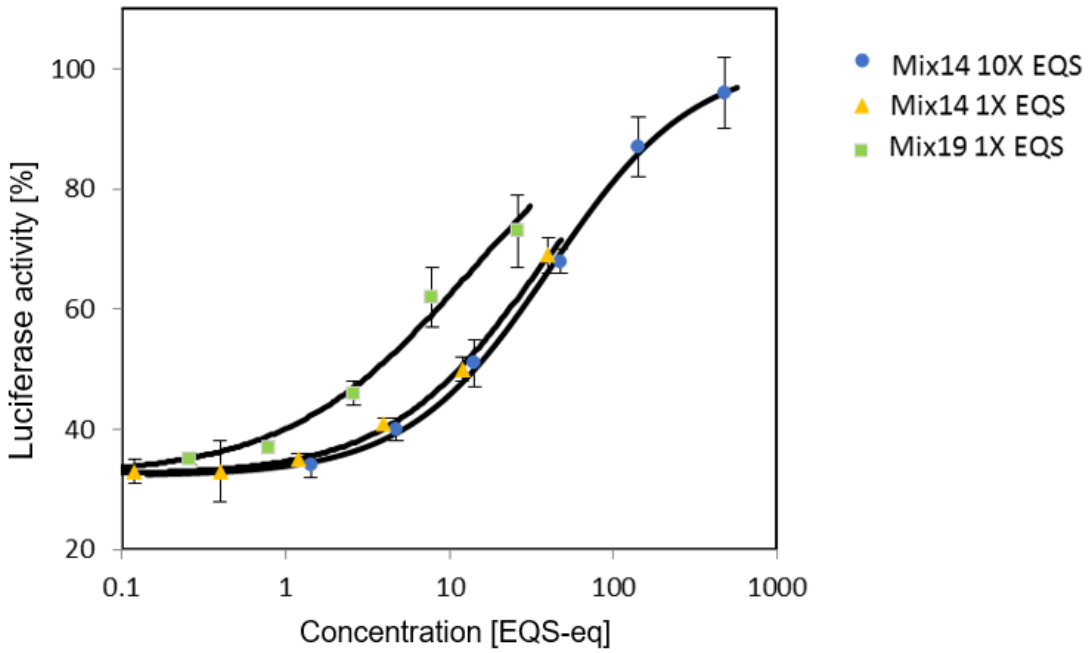


Figure 27. Concentration-dependent induction of luciferase activity by reference mixtures in MELN cells. The concentration is given as relative to the initial mixture EQS concentration and varies according to the mixture, i.e. concentration 1 = 10xEQS in Mix14 10X EQS and 1xEQS in Mix14 1X EQS and Mix19 1X EQS.

Table 36. Effective concentrations of Mix14 10X EQS, Mix14 1X EQS and Mix19 1X EQS and 17β-estradiol in the MELN assay.

Sample	EC_{50}	EC_{10}
Estradiol	12 pM	0.7 pM
Mix14 10X EQS	42.6 EQS-eq	2.7 EQS-eq
Mix14 1X EQS	39.7 EQS-eq	2.5 EQS-eq
Mix19 1X EQS	15.31 EQS-eq	0.97 EQS-eq
Solvent Control	Non active	Non active

5.4.3 ER-activated *cyp19a1b*-GFP induction in transgenic zebrafish embryos (EASZY)

INERIS

Among the three mixtures tested, Mix14 10xEQS significantly induced GFP expression (Figure 28). This up-regulation was observed from the highest dilution tested (i.e. 0.4-fold EQS-eq) but the significant effect was observed for the dilution corresponding to 4-fold EQS-eq. EE2 0.05 nM led to a tremendous induction of GFP with a mean fold of induction±sem of 26±2. No significant effect was observed for Mix14 1X EQS and Mix19 1X EQS for the dilution range tested.

Based on these data, it can be concluded that the Mix14 10X EQS contained active compounds that are able to induce an estrogenic effect in the developing brain of fish embryo by up-regulating the transcriptional activity of the ER-regulated gene *cyp19a1b*. Given that *cyp19a1b* is expressed in radial glial cells, the results also demonstrate that Mix14 10X EQS contains endocrine active substances that target these cells known to act as progenitorsof new neurons (Pellegrini et al. 2007).

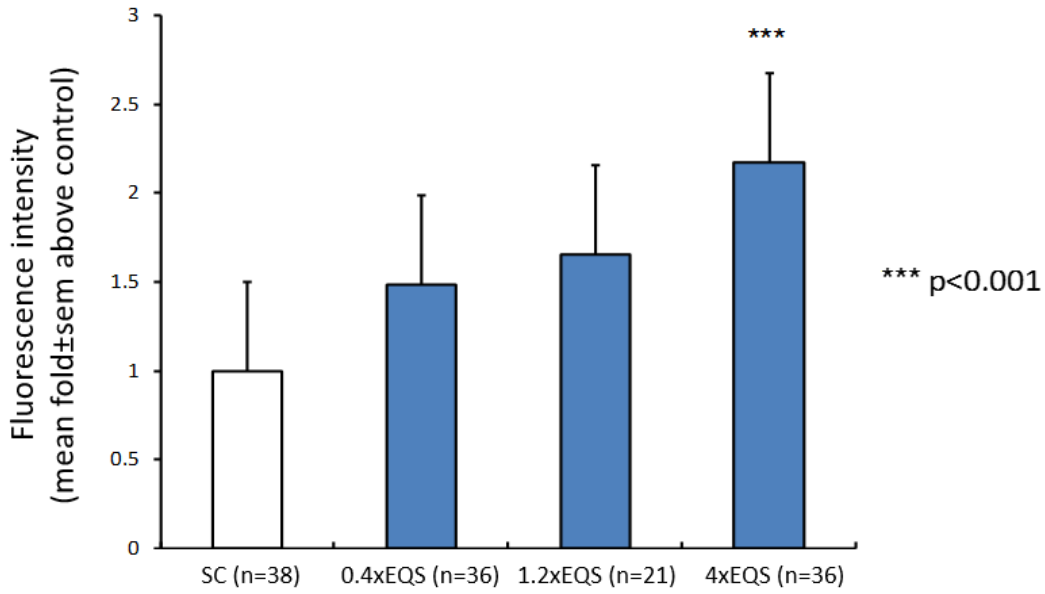


Figure 28. Effect of the Mix14 10X EQS on GFP expression in the transgenic *cyp19a1b*-GFP zebrafish embryos assay (EASZY assay). The total number of fish analysed for each treatment and the solvent control (SC) is mentioned in brackets; n = 3 independent experiments.

5.4.4 *In vitro* human ERα^{LBD} competition assay

Table 37 lists the IC_{50} values and estradiol equivalent concentrations of the mixture samples for estrogen disrupting compounds (EDCs) measured through the *in vitro* human ERα^{LBD} competition assay.

Table 37. Effective concentrations of Mix14, Mix19 and 17β-estradiol in the competition assay using the recombinant ERα^{LBD}. IC_{50} values were obtained by the average of at least four different experiments and indicate the concentration of test compound which reduces the maximum polarisation by 50%.

Sample	EEQ (ng/L)	IC_{50}
Estradiol	-	$16x10^{-9}$ M
Mix14 1X EQS	58.1	74.9 EQS-eq
Mix19 1X EQS	553.7	7.8 EQS-eq
Solvent Control	Non active	Non active

Figure 29 represents dose-dependent estrogenic activity of Mix14 and Mix19 measured in the competition assay using the recombinant ERα^{LBD}. The IC_{50} values are lower compared to YES assay but higher respect to other *in vitro* methods. This method was more sensitive to Mix19 than Mix14 being the most sensitive (after ER-CALUX) of the used methods to MIX19.

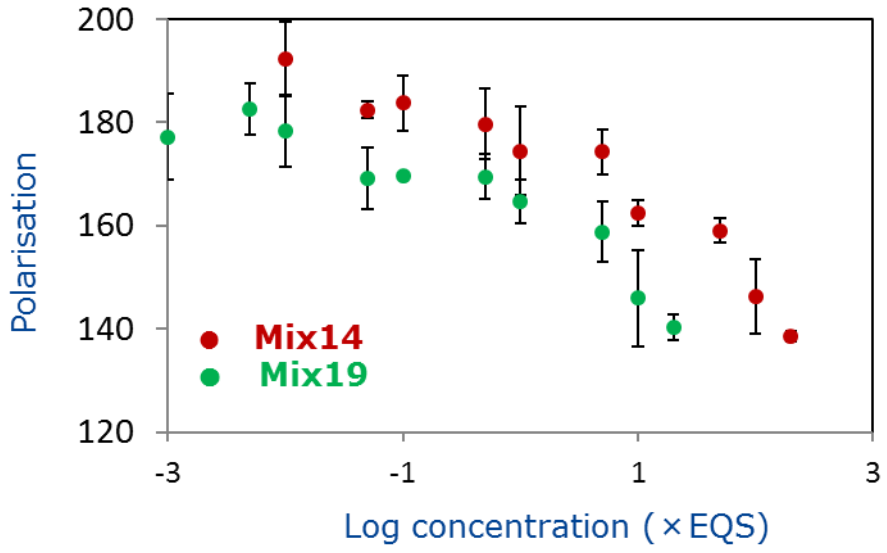


Figure 29. *In vitro* ERα^{LBD} competition assay with two different mixtures (red dots Mix14 and green dots Mix19).

5.4.5 *In vivo* exposure in fish analysis of molecular biomarkers

The highly diluted mixtures were tested at concentrations below the EQS values as follows: 0.016xEQS for Mix14 1X and Mix19 1X EQS, and 0.16xEQS for Mix14 10X EQS. No significant differences between exposed fish, contrls and untreated animals were detected neither for enzyme activity or gene expression (Figures 30 and 31).

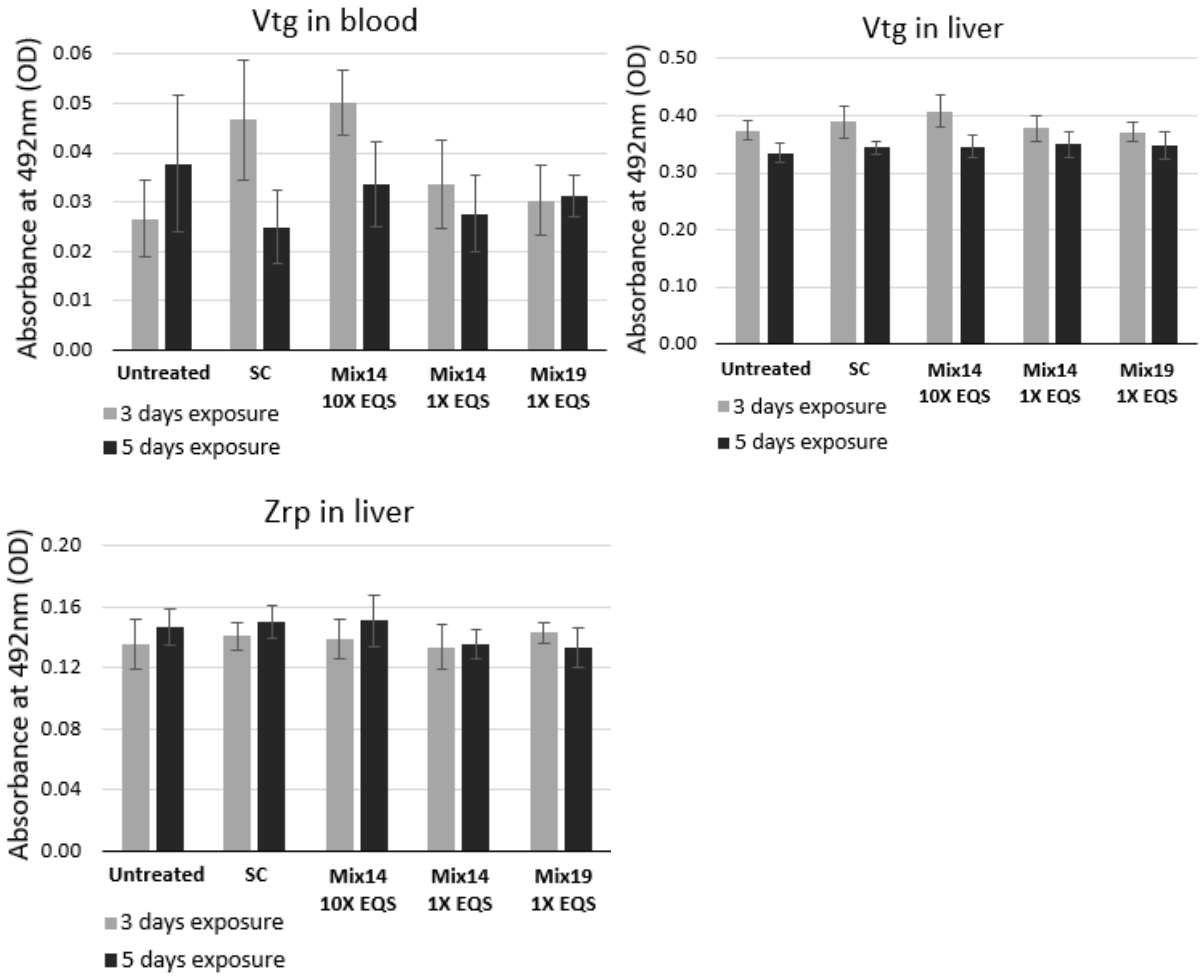
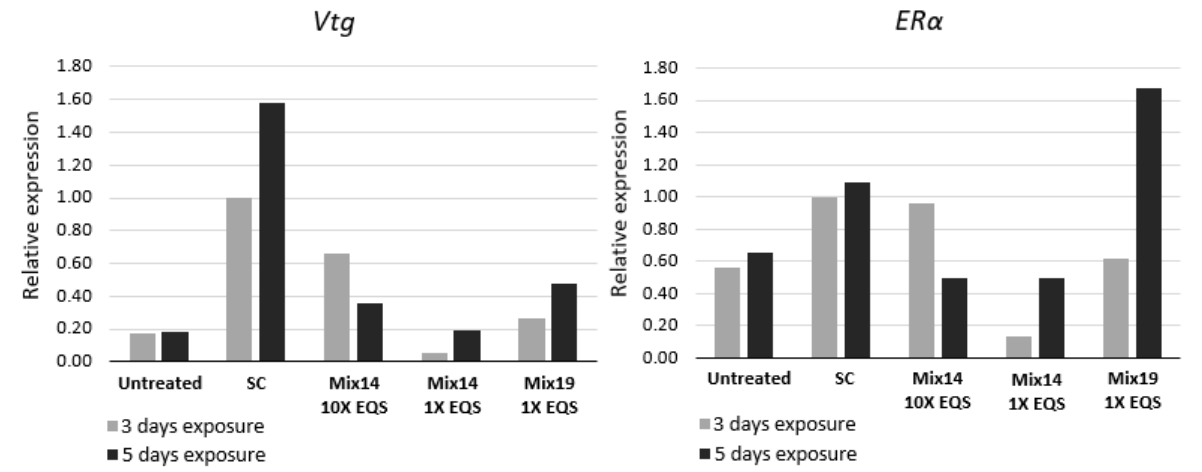


Figure 30. Enzyme activity in Atlantic salmon after exposure to chemical mixtures. Semi-quantitative ELISA was performed to assess Vtg levels in blood and liver of treated fish, and Zrp levels in liver homogenates after exposure to Mix14 10X EQS, Mix14 1X EQS or Mix19 1X EQS for 3 or 5 days. Error bars represent ±SD (n = 5).



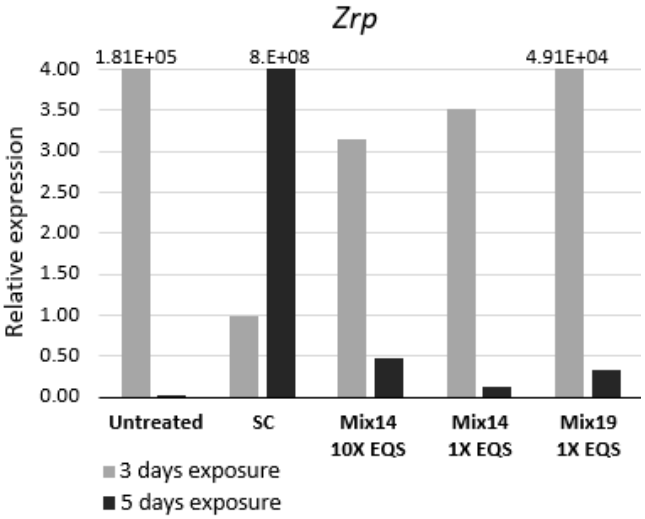


Figure 31. Relative *Vtg*, *Zrp* and *ERα* gene expression in liver homogenates of Atlantic salmon exposed to chemical mixtures. Fish were exposed to Mix14 10X EQS, Mix14 1X EQS or Mix19 1X EQS for 3 or 5 days. Total RNA was isolated for further qRT-PCR analysis. Control cells are treated with the chemical solvent solutions and the expression level in the controls is set to 1.

5.5 Androgenicity

5.5.1 Androgen receptor (AR)-CALUX®

ECOTOX

No androgenic activities were measured in the samples as well as in the solvent control and the SPE blank. Table 38 presents the effect data based on and derived from the nonlinear fit generated by GraphPad Prism 5. The EC_{50} values of the reference compound dihydrotestosterone (DHT) were in the same range as in previous studies (Sonneveld et al. 2005).

Table 38. Androgenic activities in the AR-CALUX. EC_{50} and DHT-eq of Mix14 10X EQS, Mix14 1X EQS and Mix19 1X EQS, controls and DHT. The relative enrichment factors (REF) refer to samples reconstituted in nanopure water**.**

Sample	EC_{50}	DHT-EQ±σ
Mix14 10X EQS	No effect measured up to REF 1	**-**
Mix14 1X EQS	No effect measured up to REF 1	**-**
Mix19 1X EQS	No effect measured up to REF 1	**-**
Solvent Control	No effect measured up to REF 100	-
SPE Blank	No effect measured up to REF 100	-
DHT plate 1	5.17×10^{-10} M	-
DHT plate 2	4.77×10^{-10} M	-

5.5.2 AR-activated luciferase induction in MDA-kb2 cells

RECETOX

No androgenic activities were measured in any of the three mixtures (Table 39). The reference compound dihydrotestosterone (DHT) at 10 nM was used as positive control.

Table 39. Androgenic activities using androgen-receptor activated reporter gene in MDA-kb2 cells.

Sample	Androgenic activity	DHT-EQ±σ
Mix14 10X EQS	No effect measured	-
Mix14 1X EQS	No effect measured	-
Mix19 1X EQS	No effect measured	-
Solvent Control	No effect measured	-
DHT	Yes	10×10^{-10} M

5.6 PPAR-CALUX - peroxisome proliferator-activated receptor γ2 activity

ECOTOX

Table 40 presents the effect data based on and derived from the nonlinear fit generated by GraphPad Prism 5 (for the detailed fit parameters see Table 40). The EC_{50} values of the reference compound rosiglitazone (see Figure 32 and Table 40) were in the same range as in previous studies (Gijsbers et al. 2011).

Mix19 1X EQS showed a signal at REF 100, which can be calculated in REQs (see Figure 33 and Table 40). However, given that the induction of the signal was only 3.4%, the sample should be reanalysed in a higher concentration to confirm the observed PPAR activity, as to our knowledge no such substances were present in the mixtures.

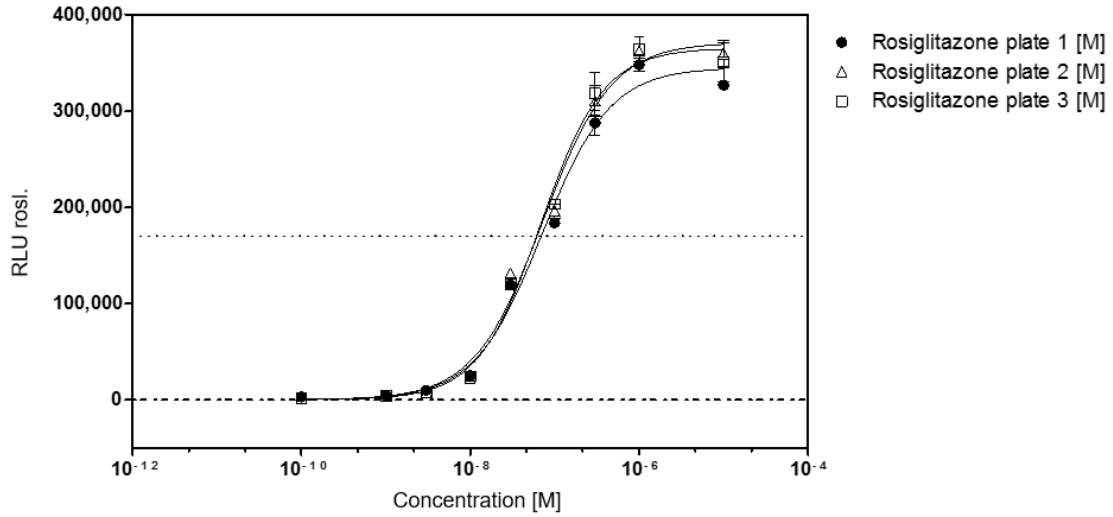


Figure 32. Induction data of the PPAR-CALUX, reference compound. Dose-response curve of the standard rosiglitazone, tested concentrations range from 1.0×10^{-5} to 1.0×10^{-10} M.

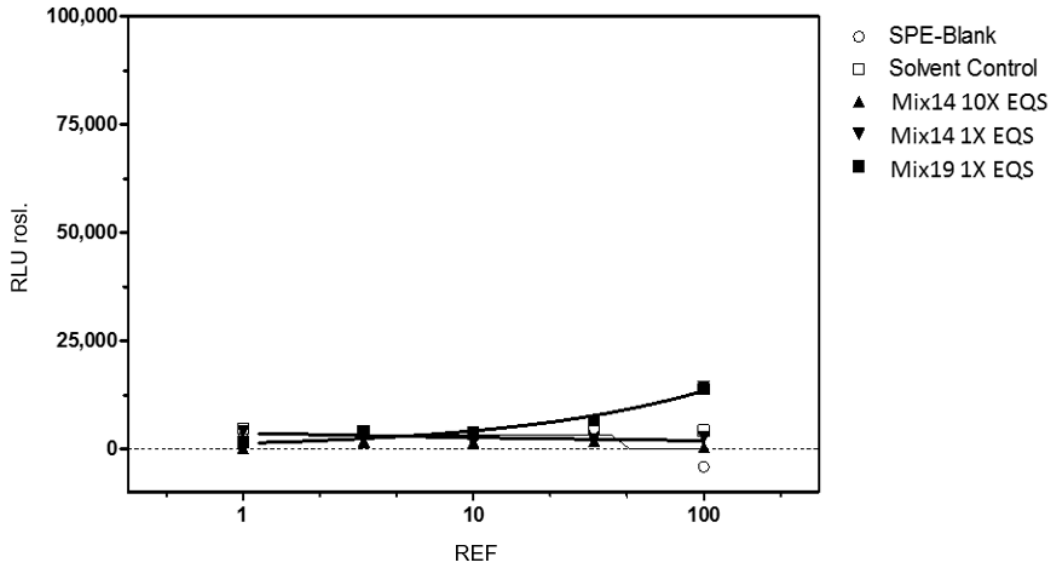


Figure 33. Activity in the PPAR-CALUX. Dose response curve of Mix14 10X EQS, Mix14 1X EQS and Mix19 1X EQS, solvent control and the SPE blank. The concentration of the samples range from REF 0.33 to 100 of the reconstituted samples.

Table 40. Induction of the peroxisome-proliferation activating receptor in the PPAR-CALUX. EC_{50} and REQ of Mix14 10X EQS, Mix14 1X EQS and Mix19 1X EQS, solvent control, SPE blank and rosiglitazone. The relative enrichment factors (REF) refer to the samples reconstituted in nanopure water.

Sample	EC_{50}	REQ±σ
Mix14 10X EQS	No effect measured up to REF 100	-
Mix14 1X EQS	No effect measured up to REF 100	-
Mix19 1X EQS	~ 9.006×10^{08} REF	15.3* ng/$L_{reconst.\ sample}$
Solvent Control	No effect measured up to REF 100	-
SPE Blank	No effect measured up to REF 100	-
Rosiglitazone plate 1	7.12×10^{-08} M	-
Rosiglitazone plate 2	7.23×10^{-08} M	-
Rosiglitazone plate 3	6.85×10^{-08} M	-

* No standard deviation because only one concentration was used for the EEQ calculation

5.7 Pregnane X receptor (PXR) activity

INERIS

PXR activity was present only in Mix14 10X EQS, but at very high concentrations, while no effect was observed at 1xEQS or 10xEQS-eq (Figure 34). The reference compound SR12813 (SR) at 100 nM was used as positive control.

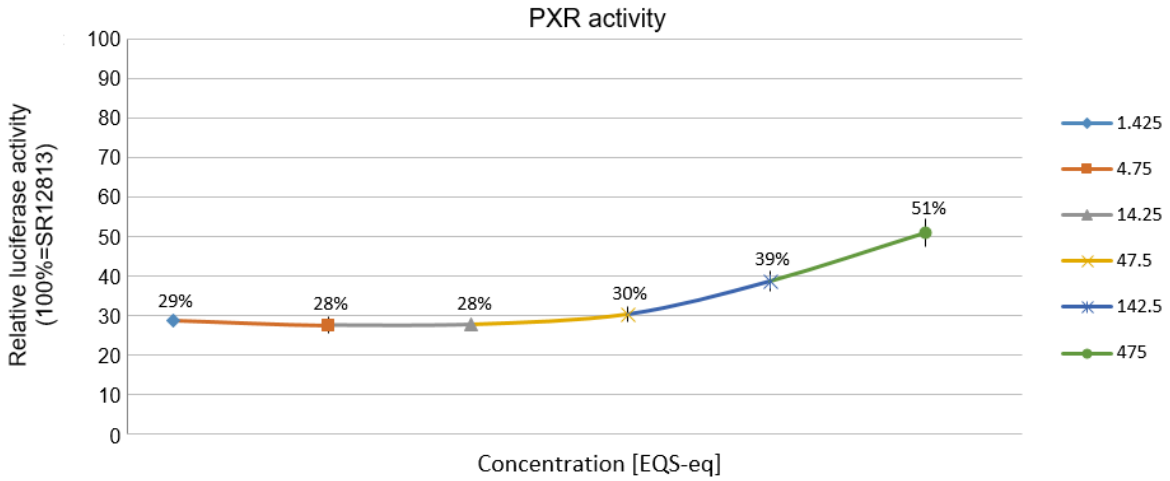


Figure 34. Induction of PXR activity by Mix14 10X EQS. Results are expressed as EQS-eq.

5.8 Aryl hydrocarbon receptor (AhR) activity

RECETOX

No activity linked to binding of the aryl hydrocarbon receptor (AhR) was observed in H4IIE-luc cells, for any of the three mixtures.

5.9 Dioxin-like activity

INERIS

No EROD induction indicating dioxin-like activity was observed in PLHC-1 cells for any of the three mixtures up to a concentration of 10xEQS-eq. The reference compound TCDD (2,3,7,8-tetrachlordibenzodioxin) at 1 nM was used as positive control.

5.10 Immunotoxicity

INERIS

The *ex vivo* impact on splenic leucocyte immune activities from the three-spined stickleback, *Gasterosteus aculeatus*, was assessed using several endpoints (Figure 35).

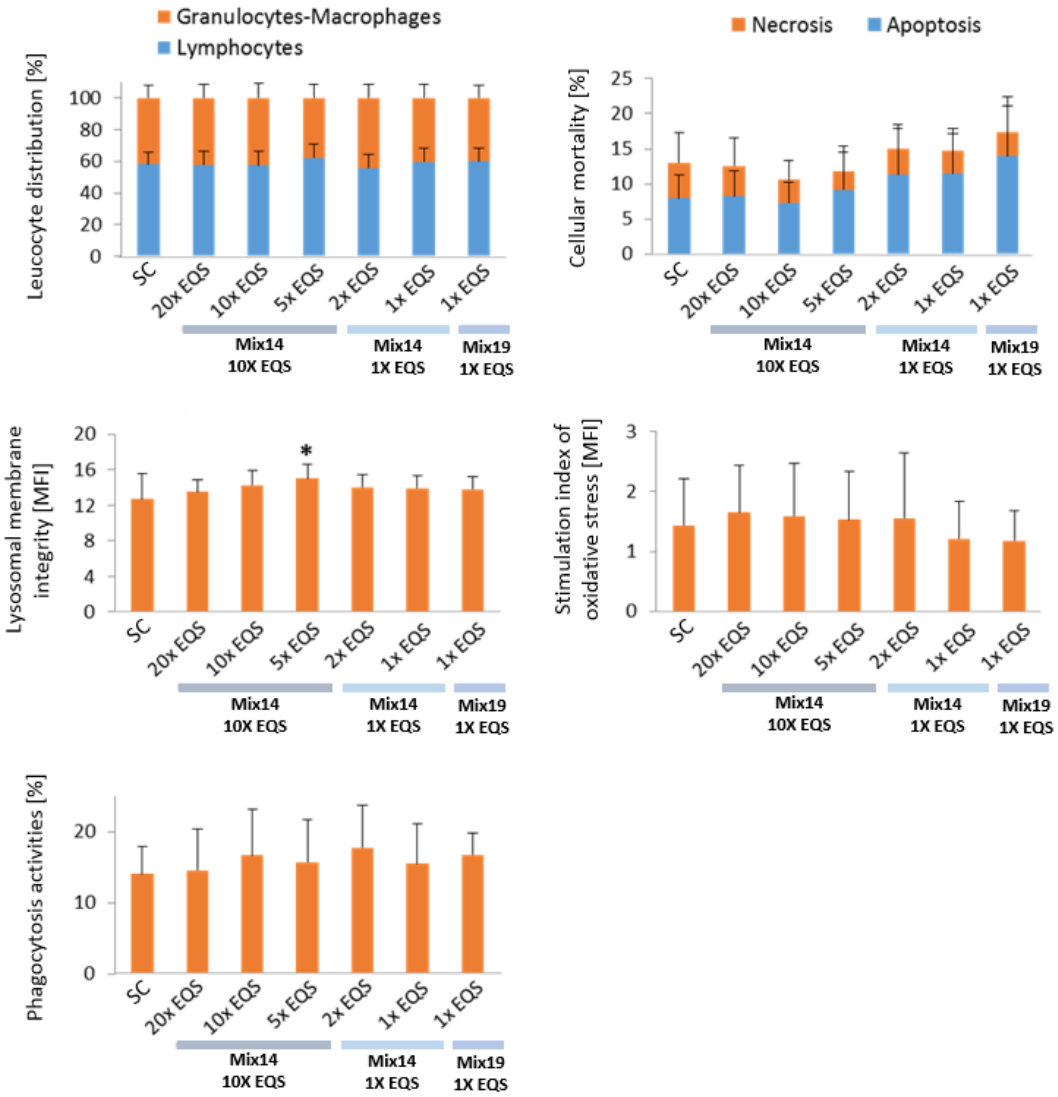


Figure 35. Mixture effects on the *ex vivo* immune activity of splenic leucocytes from *G. aculeatus*.

5.11 Developmental effects

AESD

Caenorhabditis elegans

As shown in Figure 36, a statistically significant ($p<0.05$) increase in lipid accumulation was observed with Mix14 10X EQS (compared to Mix14 1X EQS), while a slight decline in solvent control and increase in Mix19 1xEQS was apparent though statistically not robust. Pharyngeal pumping, which is under neuronal control, was statistically indistinguishable between the exposures. Nematode growth was uniform between exposures during the first 72 hours, but started to deviate at later time-points. At 120 hours, exposure to Mix19 1X EQS induced a statistically significant inhibition of development (*$p<0.05$). No statistically significant trends in movement were recorded.

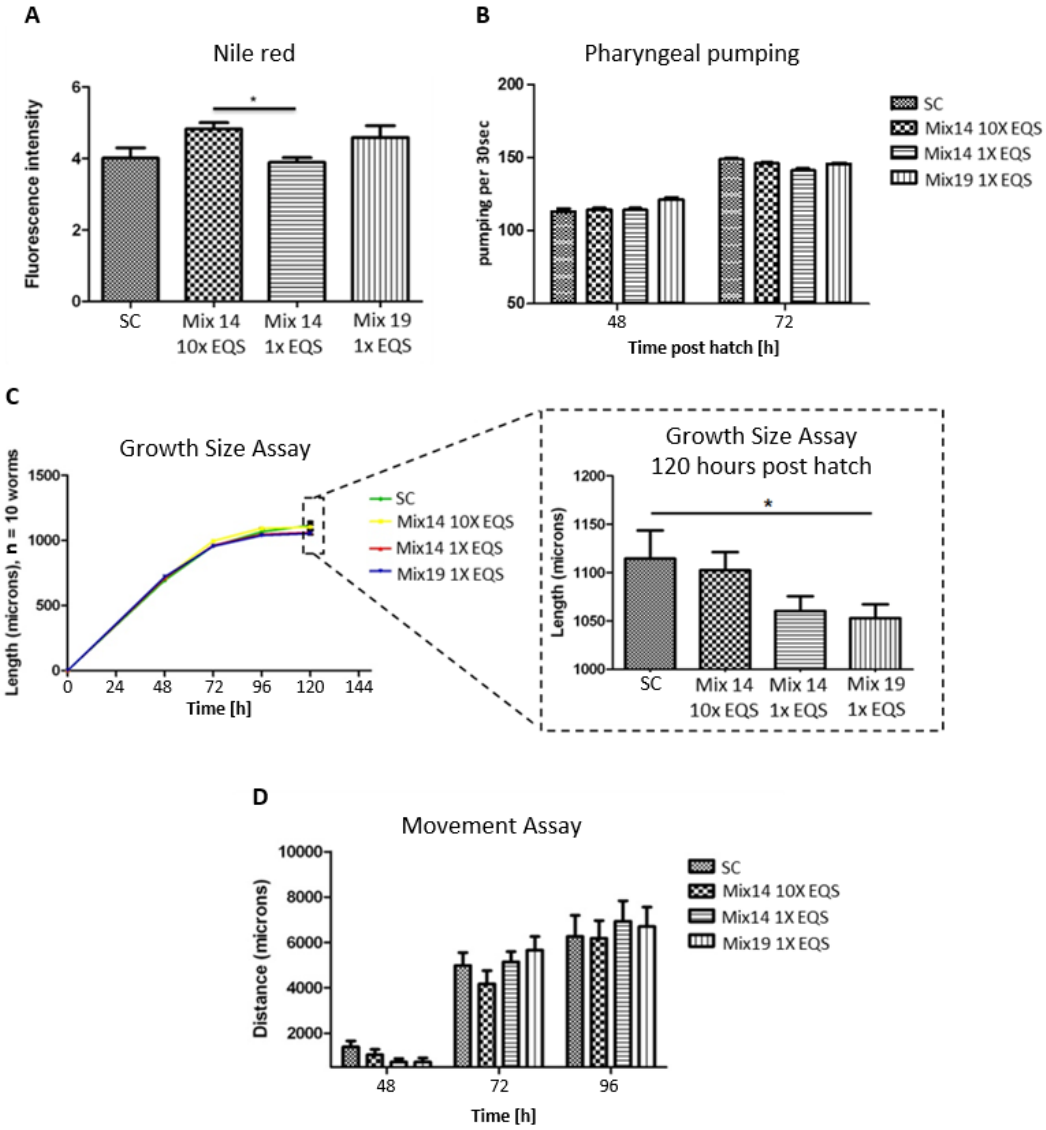


Figure 36. Nematode phenotype analyses. Nematodes were exposed to solvent control (SC) or either of the three mixtures (Mix14 10X EQS, Mix14 1X EQS or Mix19 1X EQS). (A) Following exposure for 48 hours (L1 to pre-adult L4 stage), Nile red staining was used to visualise and quantify (using Image-ProExpress, n = 10 per condition) the accumulation of lipids in storage compartments. (B) Food intake was evaluated by counting pharyngeal contractions at two time-points (n = 15 per condition), namely pre-adult L4 stage (48 hours post L1) and adults (72 hours post L1). (C) Changes in development (nematode length) was measured over time (n = 10 per condition) and (D) activity (movement) per unit time (n = 15 per condition).

5.12 Molecular biomarkers – induced expression of reporter genes in genetically modified bioluminescent organisms

5.12.1 *Escherichia coli*

HUJI

The bacterial bioreporters employed in this study are genetically engineered microbial strains, "tailored" to detect specific chemicals, groups of chemicals or global biological effects such as toxicity or genotoxicity (van der Meer and Belkin, 2010). In most cases, the engineered constructs harbor a sensing element that detects the presence of the target compound(s), fused to a reporter element, the expression of which yields a quantifiable

output. Here, the induction by the target mixtures of a panel of 12 bioluminescent bacterial reporters, each containing different sensor element, were tested.

Table 41 presents a summary of the lowest concentrations of the three samples detected by the bioreporters, and lists the reporter strains that exhibited the highest sensitivity (i.e. lowest detection threshold) to these samples. Out of the 12 reporters tested, the ones that exhibited the lowest detection thresholds were *zntA* (Mix14 10X EQS and Mix14 1X EQS), *arsR* (Mix14 10X EQS), *cydA* (Mix14 1X EQS and Mix19 1X EQS) and *micF* (Mix19 1X EQS). As is apparent from the stress-response characteristics of these gene promoters (Table 41), their induction indicates the presence of heavy metals in Mix14 10X EQS and Mix14 1X EQS, respiration inhibition in Mix14 1X EQS and Mix19 1X EQS, and oxidative-stress inducing chemical(s) in Mix19 1X EQS.

Table 41. Bioluminescent reporter strains induced and the highest concentration detected.

Sample	Lowest concentration detected (relative to EQS)	Most sensitive bioreporters (gene promoters induced)	Indicated stress
Mix14 10X EQS	0.625x	*zntA*+*arsR*	Heavy metals
Mix14 1X EQS	2.5x 1.25x	*zntA* *cydA*	Heavy metals Respiratory inhibition
Mix19 1X EQS	0.156x 5x	*micF* *cydA*	Oxidative damage Respiratory inhibition

Figure 37 presents the dose-dependent induction of the *zntA::lux* and *arsR::lux* reporters by Mix14 10X EQS. The data are presented in two modes – either as the ΔRLU values (RLU of the sample minus RLU of the control, Figure 37A) or as the response ratio (RLU of the sample divided by the RLU of the control, Figure 37B). The response ratio kinetics of the two reporters indicates that a response (ratio ≥ 2) was obtained already after 10-20 minutes of exposure (data not shown).

As Mix14 1X EQS is a x1/10 dilution of Mix14 10X EQS, *zntA::lux* that was induced by Mix14 10X EQS was also induced by Mix14 1X EQS (Figure 38A-B). The response of the *arsR::lux* reporter was minimal (data not shown). Interestingly, a late response to Mix14 1X EQS was observed in *cydA::lux*, a reporter for respiratory inhibition (Figure 38C). It is possible that the more concentrated mixture Mix14 10X EQS inhibited the induction of *cydA* sensing element.

Mix19 1X EQS is identical to Mix14 1X EQS, with the addition of 5 more chemicals (Table 1). The *micF::lux* reporter, induced by cellular oxidative stress, was induced by a very low concentration (0.156xEQS) as can be observed in Figure 39. It can also be observed that higher concentrations (2.5-5xEQS) inhibited the sensing element induction. The response of *cydA::lux* to Mix14 1X EQS was similar to that of Mix19 1X EQS (data not shown).

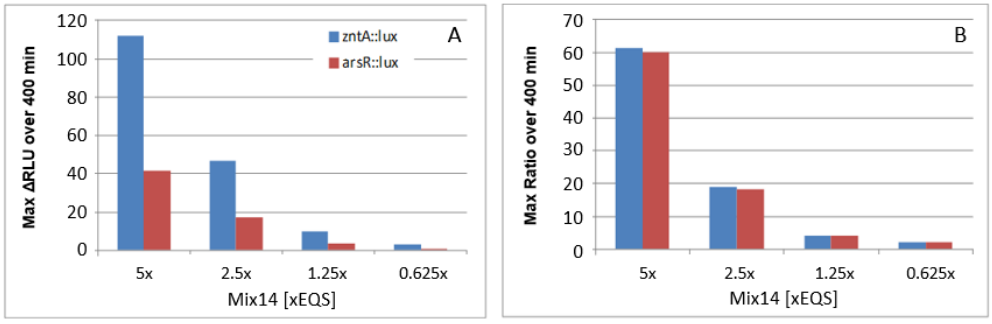


Figure 37. Induction of *zntA* and *arsR* by Mix14 in *E. coli*. Dose-dependent luminescent response. (A) Luminescence intensity (ΔRLU). (B) Response ratio.

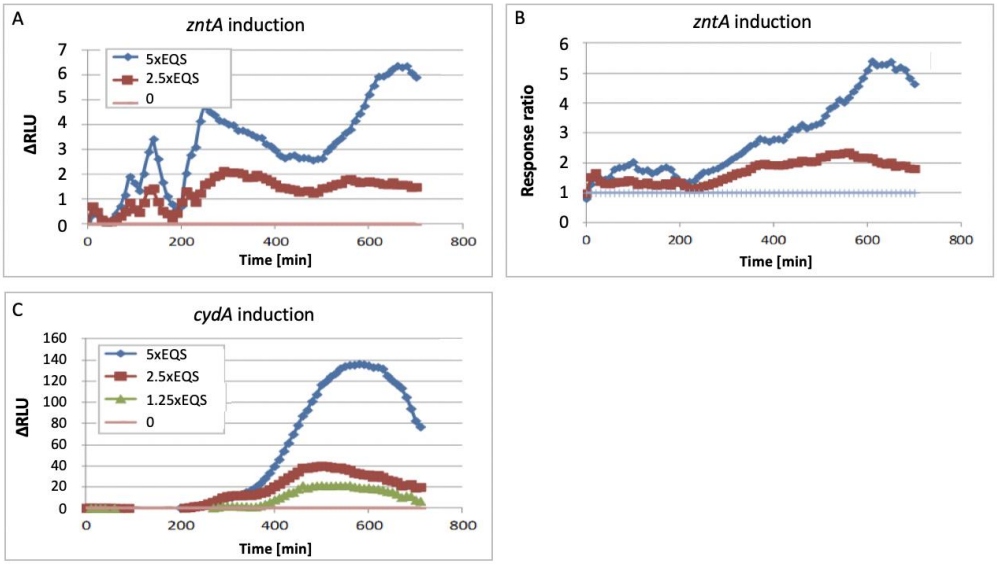


Figure 38. Induction of *zntA* or *cydA* by Mix14 in *E. coli*. Dose-dependent luminescent response. Time course of luminescence signal development is presented as (A) luminescence intensity (ΔRLU) and (B) response ratio for *zntA::lux*, and as (C) luminescence intensity (ΔRLU) for *cydA::lux*.

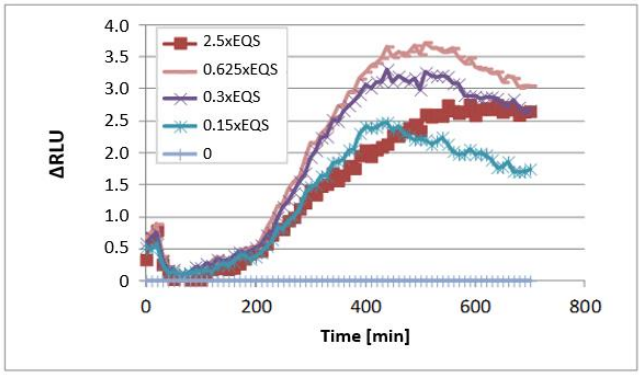


Figure 39. Induction of *micF* by Mix19 1X EQS in *E. coli*. Dose-dependent luminescent response. Time course of luminescence signal development of *micF::lux.*

5.12.2 *Saccharomyces cerevisiae*

JRC

The mixtures were analysed regarding to growth, genotoxicity and acute toxicity effects in yeast. 4-NQO was used as positive controls, as it is a known genotoxic compound in mammals.

5.12.2.1 Effect on yeast growth

4-NQO induced a dose-dependent growth inhibition in all the yeast strains, which was higher than 20% already at 5 µg/L. The effect in strains 261 and 545 is shown in Figure 40. Regarding the exposure to Mix14 10X EQS and Mix19 1X EQS, none of the concentrations used in this study showed significant (>20%) growth inhibition (Figure 40). The maximum effect observed was of 8.89±2.1% growth inhibition at 250-fold EQS in Mix14 10X EQS. A similar effect was observed for strains 545 and 549 when exposed to 4-NQO or the mixtures (data not shown).

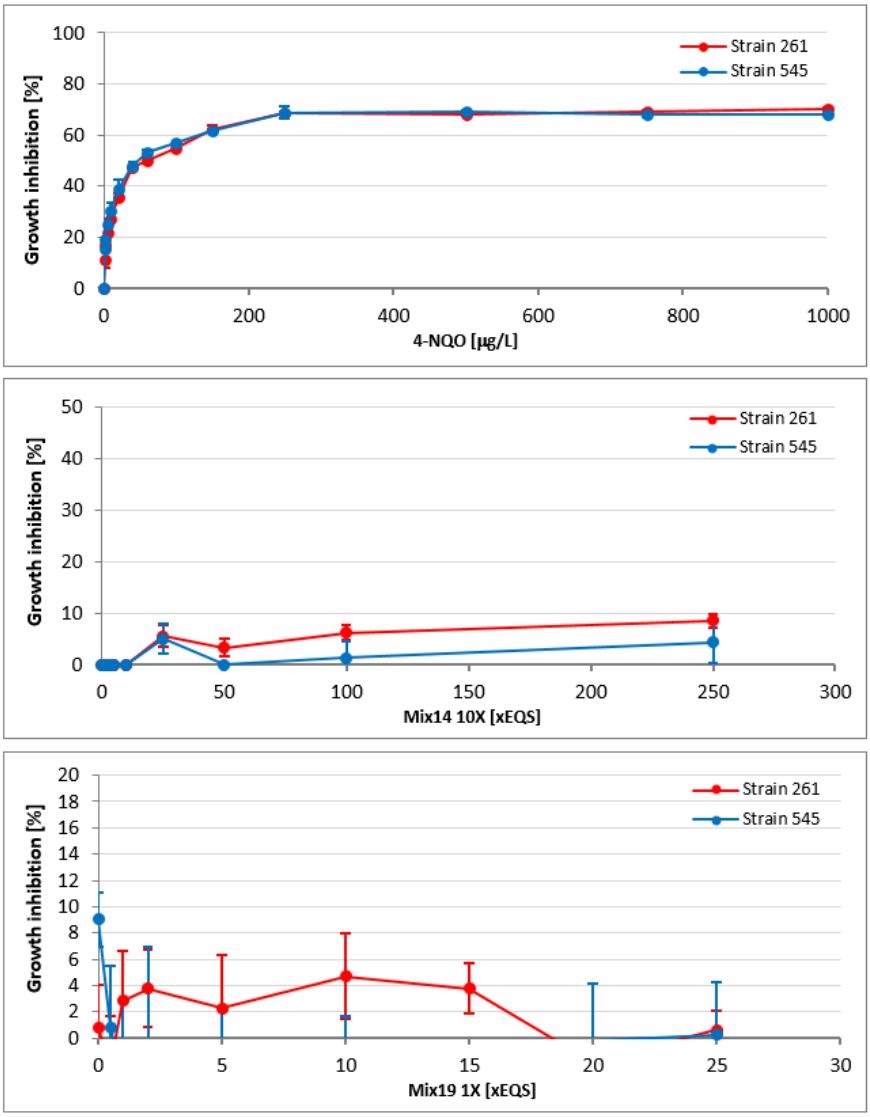


Figure 40. Growth inhibition of yeast strains (261 and 545). The strains were exposed to the positive control 4-NQO (top) and different concentrations of Mix14 10X EQS (middle) and Mix19 1X EQS (bottom). Data are presented as mean±SD (n = 24).

5.12.2.2 Effect on genotoxicity

The genotoxic compound 4-NQU induced the expression of *RAD54*-GFP (reporter for DNA integrity damage), with an induction ratio (IR) higher than 1.4 (threshold value for genotoxicity) already at 20 µg/L (Figure 41), thus confirming its suitability as positive control in this system. The IR increased in a dose-dependent manner in strain 261 (indicator for genotoxicity) meanwhile in strain 545 no induction was observed (control strain for genotoxicity). The genotoxicity observed by exposure to different concentrations of mixtures is shown in Figure 41. None of the concentrations used in this study showed significant genotoxicity, with IR above 1.4 according to Schmitt et al. (2005).

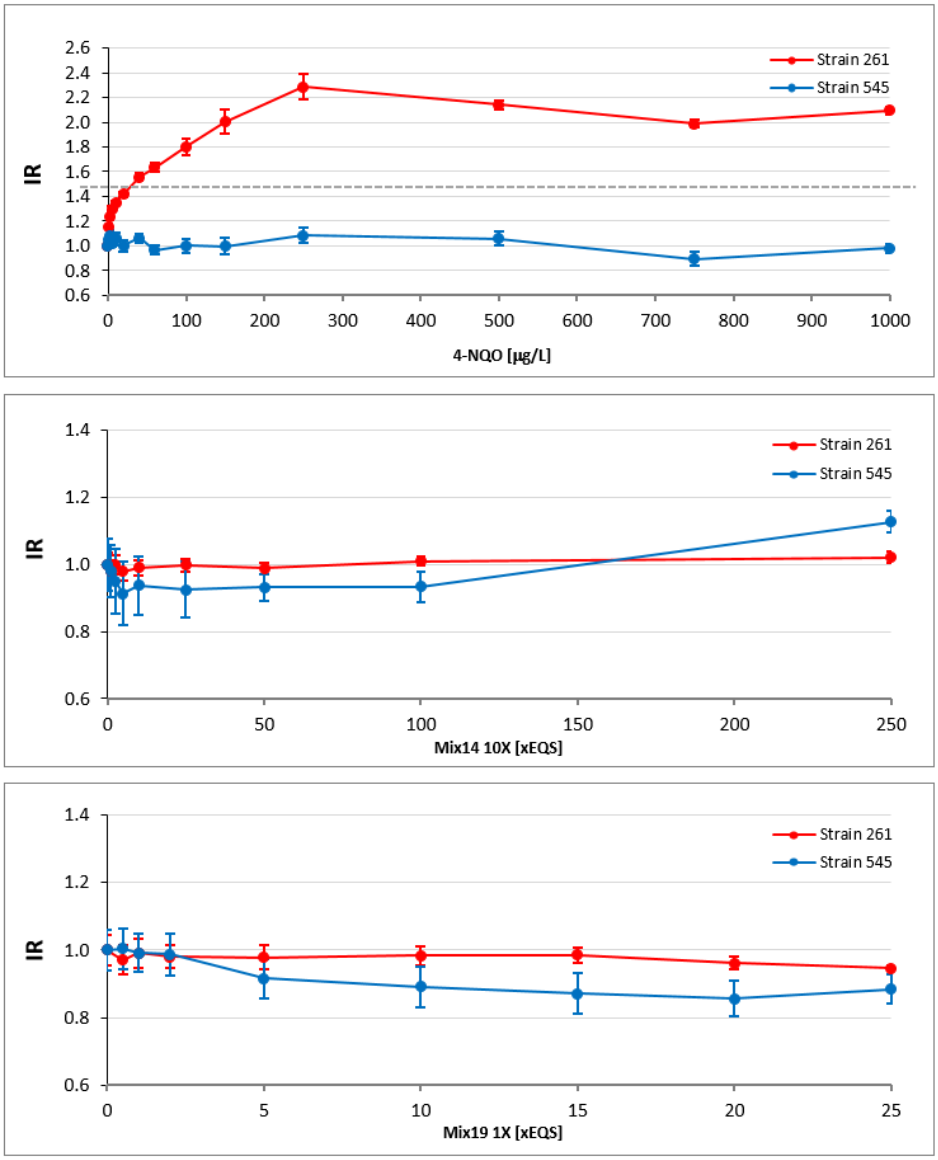


Figure 41. Induction of the *RAD54*-GFP expression in yeast. The yeast strains 261 (indicator) and 545 (control strain) were exposed to the positive control 4-NQO (top), to Mix14 10X EQS (middle) and Mix19 1X EQS (bottom). Data are presented as mean±SD (n = 24).

5.12.2.3 Effect on acute toxicity

The acute toxicity was measured by the reduction of IR linked with the decrease in the PPMA1-mediated transcriptional activation of the yeast optimised green fluorescent protein (yEGFP3). The acute toxicity effects of exposure to the positive control compound 4-NQU is shown in Figure 42, by the reduction of IR in strain 544 (indicator) vs. strain 549 (control). The dose-dependent toxicity was significant ($p<0.05$) only after exposure to 25-fold EQS equivalent concentrations as shown in Figure 42. None of the concentrations of Mix19 1X EQS used in this study showed significant acute toxicity.

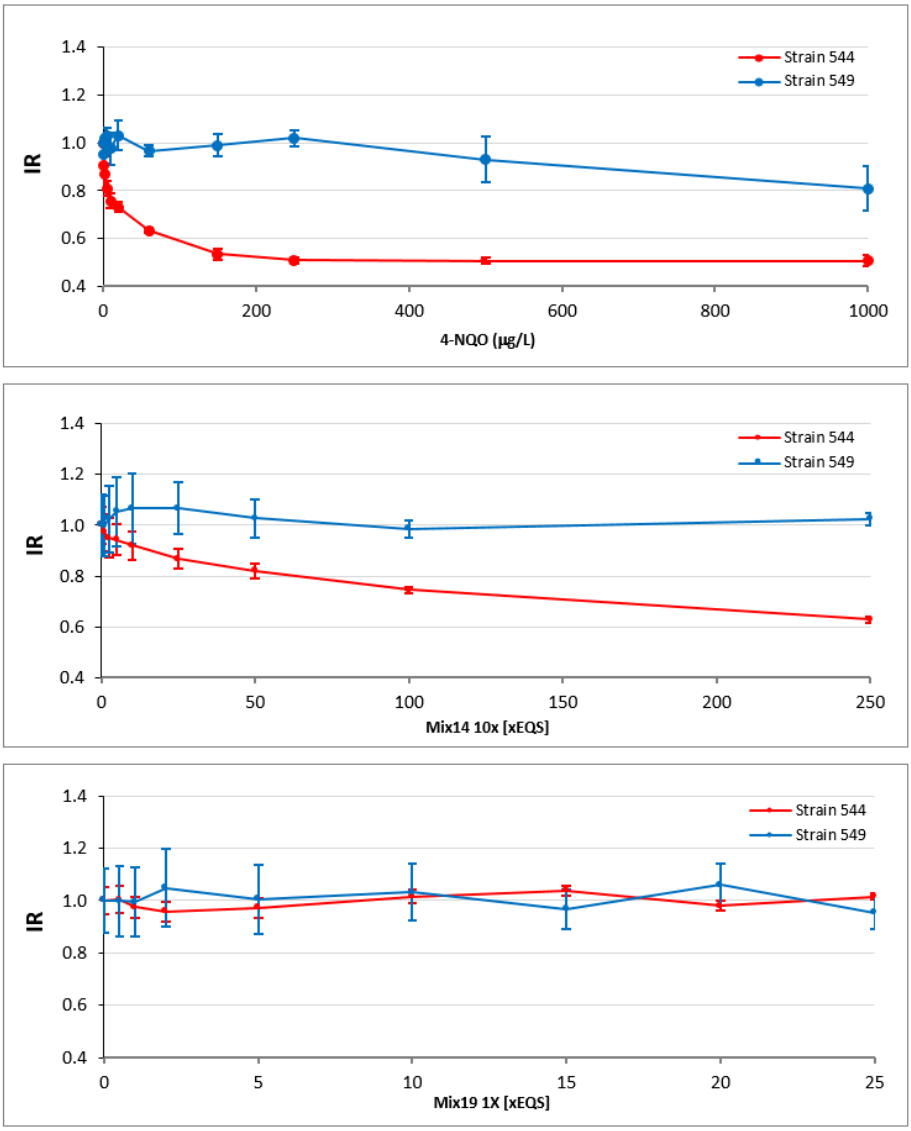


Figure 42. Acute toxicity of yeast strains (544 and 549). Yeasts were exposed to the 4-NQO (top panel) and to different concentrations of Mix14 10X EQS (middle panel) and Mix19 1X EQS (bottom panel). Data are presented as mean ± SD (n=24).

5.12.3 *Caenorhabditis elegans*

AESD

Cyp-35a2 fluorescence was sufficiently sensitive to distinguish between the three mixtures, but *mtl-2* only at the 10-fold concentration (Figure 43). In contrast, *gst-38* was statistically significant only in Mix19 1X EQS and no statistical difference was observed with *ugt-1* and *gcs-1* at the doses tested.

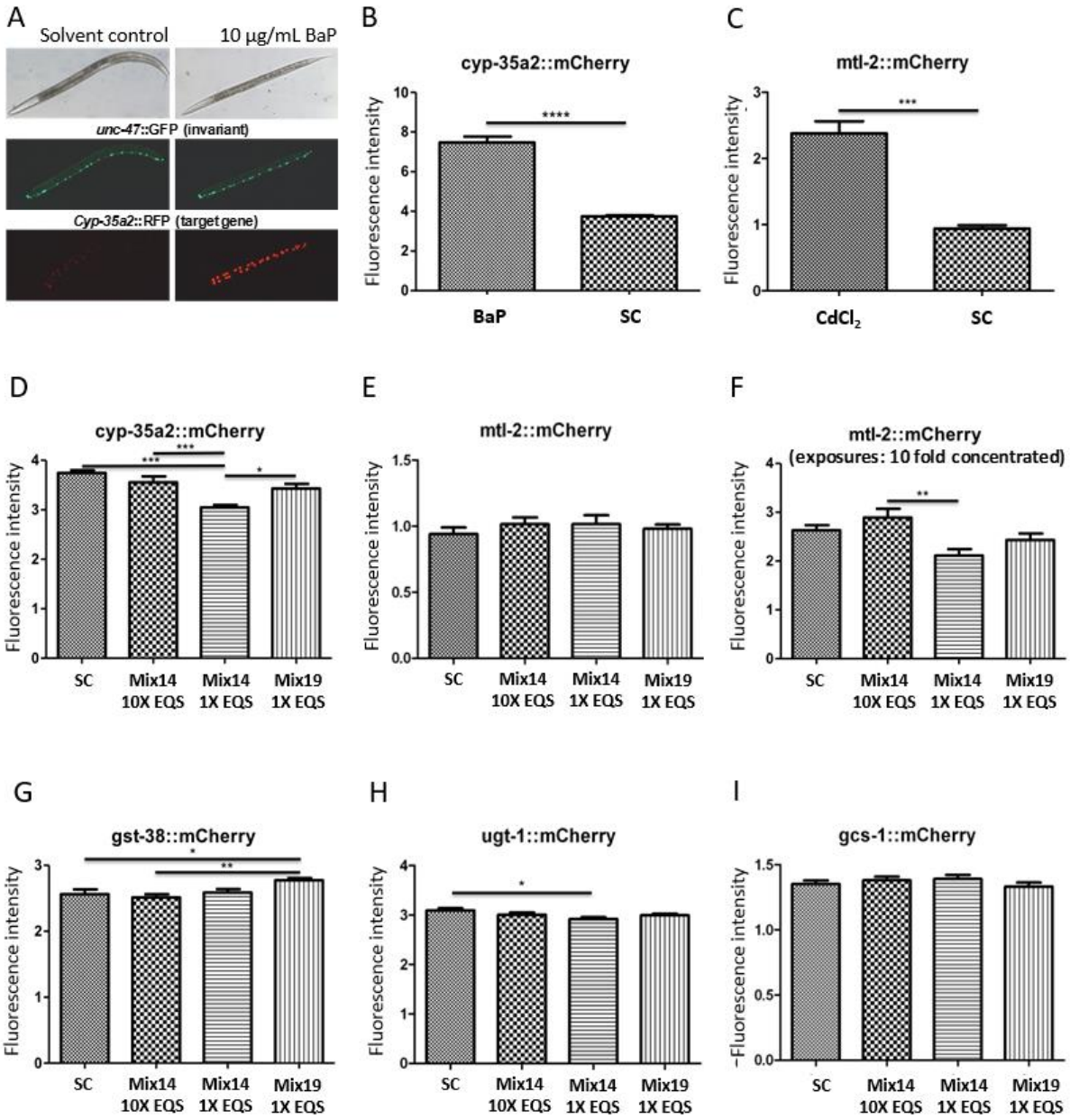


Figure 43. Nematode fluoromics. Representative example of a dual-fluorescent nematode (*Caenorhabditis elegans*) expressing, *in vivo*, a green fluorescent protein (GFP) which is driven by the promoter of the invariant *unc-47*, a transmembrane vesicular GABA transporter, and a red fluorescent protein (mCherry) induced by the promoter of *cyp-35a2*, a benzo[a]pyrene (BaP) exposure-responsive transcript (A). Fluorescence (mCherry signal normalised to GFP signal) was quantified using ImageJ (n = 10 per condition). Two positive controls included *cyp-35a2* following exposure to BaP (100 µg/mL) (B) and the metal responsive *mtl-2* following exposure to Cd (4 mg/mL) (C). A suite of age synchronous transgenic nematodes was exposed to solvent control (SC), Mix14 10X EQS, Mix14 1X EQS or Mix19 1X EQS from L1 to pre-adult L4 stage (48 hours

chronic exposure) and the expression of the following genes was evaluated: *cyp-35a2* (D)*; mtl-2* (E)*; mtl-2* at a dose 10-fold more concentrated (F); *gst-38,* a glutathione-S-transferase involved in phase II detoxification (G); *ugt-1,* a phase II (conjugative) UDP-glucuronosyltransferase (H) and *gcs-1,* a γ-glutamine cysteine synthetase heavy chain (*GCS(h)*) which functions in a conserved oxidative stress response pathway as a phase II detoxification enzyme by catalysing the rate-limiting first step in glutathione biosynthesis (I). Note that *cyp-35a2* fluorescence was sufficiently sensitive to distinguish between the three mixes, but *mtl-2* only at the 10-fold higher concentration. In contrast, *gst-38* was statistically significant only in Mix19 1X EQS and no statistical difference was observed with *ugt-1* and *gcs-1* at the doses tested. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

5.12.4 Cell lines - gene expression analysis by qPCR

ORU

All cells treated with the solvent solutions (methanol and HNO_3) showed no difference in gene expression compared to untreated cells (not shown).

Since Mix14 10X EQS is composed of the same 15 substances as Mix14 1X EQS, but at 10 time's higher concentration (see Table 5), it was expected that exposure to these two mixtures would result in a dose-response pattern. However, as demonstrated by the qPCR data, exposure of HeLa cells to the mixtures did not show the expected trends (Figure 44). A down-regulation of the androgen receptor (AR), metallothionein (MT), glutathione S-transferase kappa1 (GSTK1), interleukin-8 (IL-8) and *p53* was registered following exposure to Mix14 10X EQS and Mix14 1X EQS. The only up-regulated gene was interleukin-6 (*IL-6*). Exposure to Mix19 1X EQS resulted in abrogation of the responses seen following exposure to Mix14 1X EQS except for *IL-6*, for which the response was increased by the addition of the 5 chemicals.

Metal regulation

Metallothionein is a protein involved in metal regulation and HeLa cells exposed to Mix14 10X EQS and Mix14 1X EQS showed a down-regulation of the *mt2A* mRNA by almost 50% (Figure 44). This suggests an effect of the chemical mixture in metal regulation.

Phase II metabolism

GSTK1 is involved in phase II metabolism and the relative gene was down-regulated by Mix14 10X EQS and Mix14 1X EQS in HeLa cells, suggesting an effect of the mixtures on this process (Figure 44). For LMH, the transcript of *CYP1A1* was expressed at insufficient levels to permit a robust analysis (Figure 44), however this gene was not affected in either HeLa or ZFL (Figures 44 and 46).

Immune response

Three genes involved in the immune system (*COX2*, *IL-6* and *IL-8*) were studied in HeLa cells. *COX2* was not affected by the chemical treatment in any of the cell lines. Low-dose exposure to the chemical mixtures up-regulated the pro-inflammatory cytokine *IL-6* in HeLa cells. However, this was not observed at the higher dose in Mix14 10X EQS (Figure 44). The only effect Mix19 1X EQS elicited in this study was the transcriptional induction of *IL-6* in HeLa cells (Figure 44). In contrast, the expression of the other pro-inflammatory cytokine *IL-8* was reduced by both Mix14 10X EQS and Mix14 1X EQS, but not by Mix19 1X EQS in HeLa cells. Mix14 10X EQS also down-regulated *IL-8* in LMH cells (Figure 45). These results suggest that there is an effect on inflammatory mediators following exposure to the mixtures.

Cell cycle

p53 encodes a cell-cycle regulatory protein allowing the cell to repair DNA but can also initiate apoptosis. This gene was down-regulated by both Mix14 10X EQS and Mix14 1X EQS in HeLa cells, however no effect on its expression from either of the mixtures was observed in LMH or ZFL.

Comparison between cell lines

In this short-time study, the chicken and zebrafish cell lines were less sensitive to the chemical mixtures than the human cell line. *IL-8* showed a small down-regulation in LMH cells (Figure 45) while none of the tested genes responded to the exposures of the ZFL cells (Figure 46). However, it remains unclear whether this difference is caused by species variances or if it is due to different cell types as HeLa cells is a epithelial cell line originary from a cervix cancer while LMH and ZFL are epithelial liver cells which may be more resistant to a variety of chemicals.

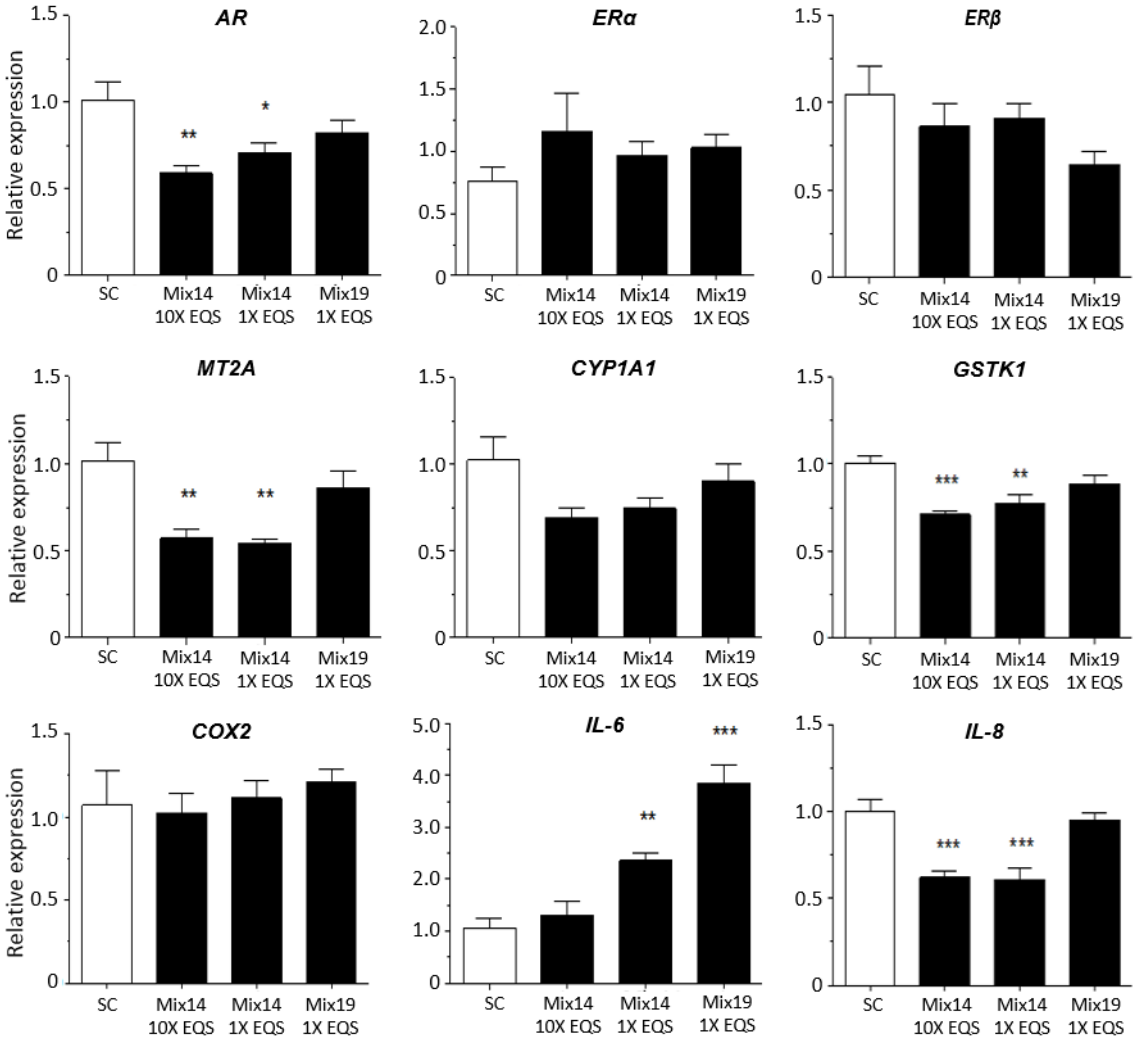


Figure 44. Relative gene transcription in HeLa cells exposed to chemical mixtures. Cultured HeLa cells were treated with Mix14 10X EQS, Mix14 1X EQS or Mix19 1X EQS for 24 hours. Total RNA was isolated for further qRT-PCR analysis. Control cells are treated with the chemical solvent solutions and the expression level in the controls is set to 1. The relative expression levels of 10 genes normalised against elongation factor 1a are shown. Statistical data analysis was performed through ANOVA followed by Dunnett's post hoc test (*p<0.05; **p<0.01; ***p<0.001). Error bars represent ±SD (n = 4).

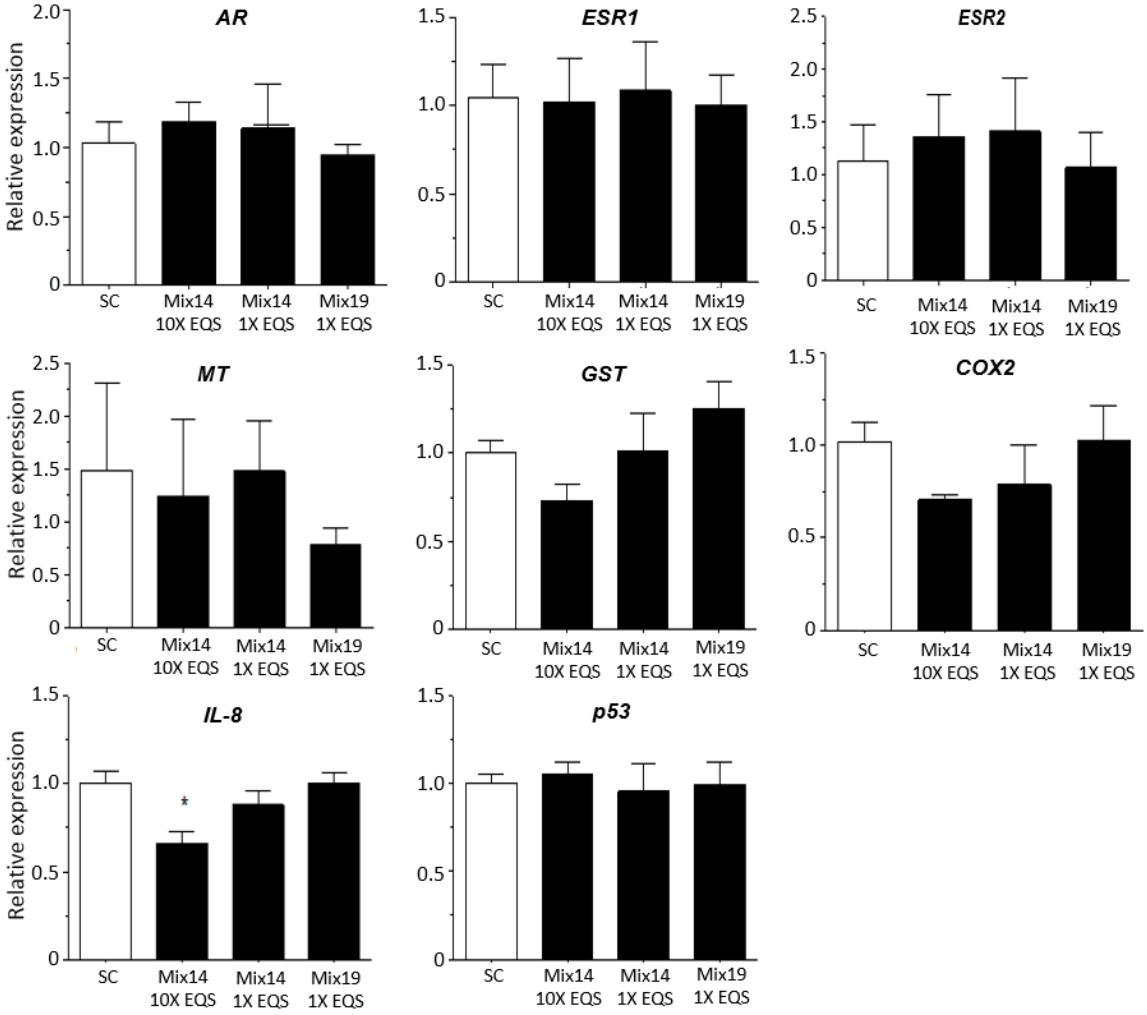


Figure 45. Relative gene transcription in LMH cells. Chicken LMH cells were treated with the chemical mixtures for 24 hours and gene expression was determined by qRT-PCR analysis. Control samples are cells treated with the chemical solvents and gene transcription is normalised against elongation factor 1a. The relative expression levels of 8 genes are shown. ANOVA followed by Dunnett's post hoc test was used to determine statistical significance (*p<0.05). Error bars represent ±SD (n = 4).

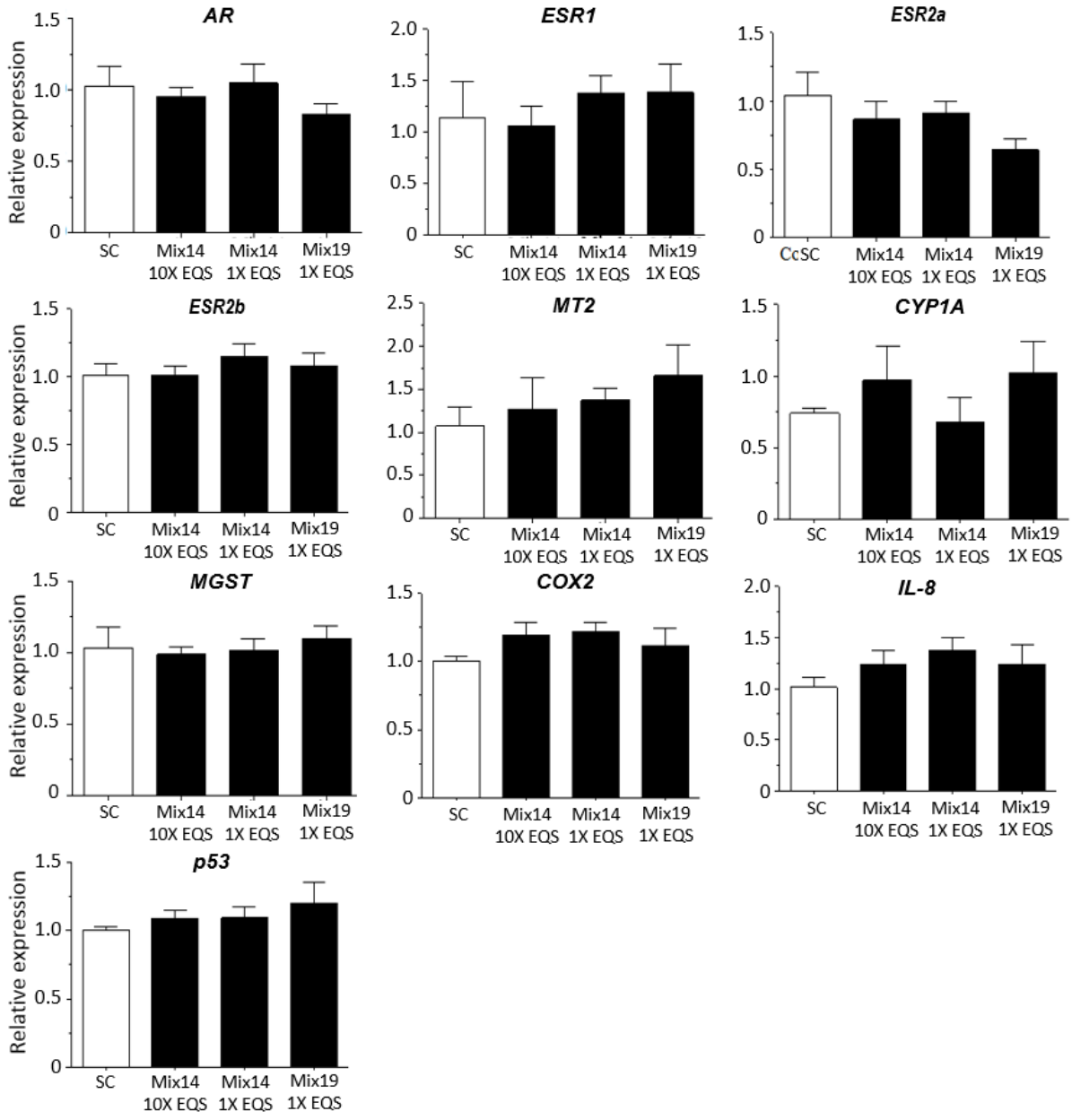


Figure 46. Relative gene transcription in ZFL cells. Zebrafish cells were exposed to the reference mixtures for 40 hours and gene expression was determined by qRT-PCR analysis. Controls: cells treated with the chemical solvents. Gene transcription is normalised against elongation factor 1a. The relative expression levels of 10 genes are shown. ANOVA followed by Dunnett's post hoc test for multiple group comparison was used to determine statistical significance. Error bars represent ±SD (n = 4).

6. DISCUSSION

The effects of chemical mixtures on water ecosystems and human health reported over recent years by the scientific community raised the attention of the European Commission (SCHER, SCENIHR and SCCS, 2013).Joint Research Centre, as the scientific and technical centre of the Commission, committed an EU-wide exercise to investigate the impact of interacting compounds on different trophic levels (Figure 47) through effect-based methods (EBMs). Artificial mixtures composed of substances representative of the most common biological effects observed in water bodies were used to reduce the uncertainty due to complex environmental samples. Determination of cause-effect associations was facilitated by known concentrations of each substance with reference to environmental quality standards (EQS) considered as safe for wildlife.

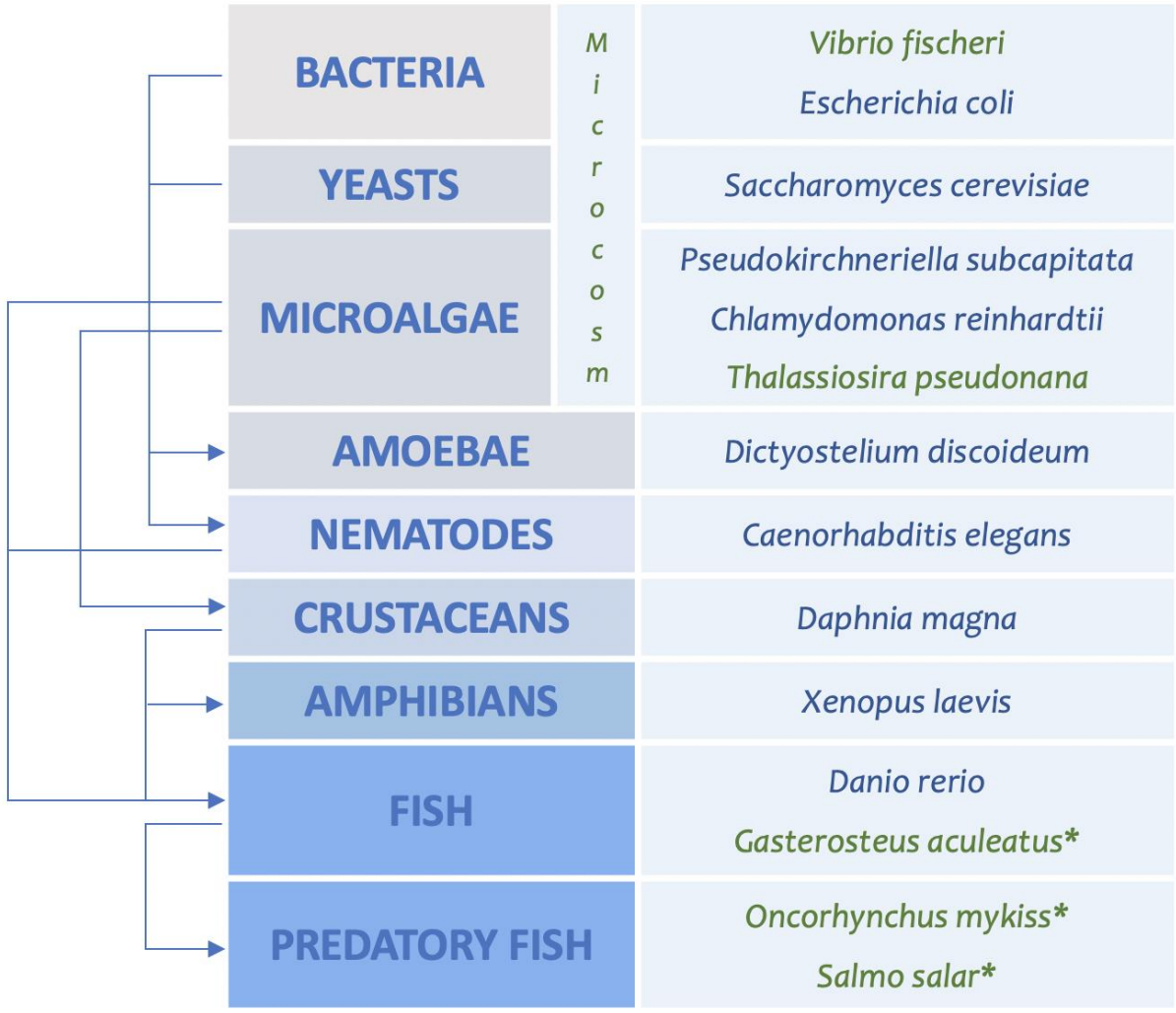


Figure 47. Taxa included in the employed bioassays. Trophic interactions are indicated by arrows directed from lower trophic levels to respective predators. Organism names are specified for prevalently freshwater (blue) and marine (green) species. * Species adapted to marine or freshwater environments during periods of the lifespan.

A battery of ecotoxicity bioassays covering different levels of biological complexity, from molecular (e.g. immune activity tests) to whole organism (e.g. fish embryo development tests) and community (microcosm study), showed relevant effects of chemical mixtures even when individual substances were present at concentrations below the EQS. Effects of reference mixtures at 1xEQS were observed across a wide range of taxa including

bacteria, algae, nematodes, amphibians and fish. These results demonstrate that health of biota may not be sufficiently protected when multiple chemicals co-occur in realistic scenarios. Further studies performed using transcriptomics tehcniques to investigate the effects at molecular level induced by chemical mixtures on microalgae will be published.

Increased effects of chemicals acting through the same pathway in a multiple-component sample reflect the combined toxicity arising from the concentration addition concept and may significantly affect aquatic organisms (Broderius, 1990; Napierska et al. 2018).

This was corroborated for the mixture of four herbicides (atrazine, diuron, isoproturon and simazine) acting as photosystem II (PSII) inhibitors in the algae toxicity test (section 5.2.2.2) and for endocrine disrupting compounds (17β-estradiol, 4-nonylphenol and bisphenol A) which bind to the estrogen receptor and activate the expression of reporter genes (section 5.4).

In a similar manner, co-occurring substances may imbalance the entire ecosystem as a consequence of effects elicited in sensitive and ecologically relevant organisms. Several bioassays confirmed such outcomes in both chronic and acute toxicity tests when each substance in the reference mixture was present at annual average (AA-EQS) and maximum allowable (MAC-EQS) concentrations, which according to the European legislation are expected to protect biota from chronic and acute exposure, respectively.

At the base of the food chain, mixtures at EQS equivalent concentrations reduced the phytoplankton community and induced an increase in the bacterial population. The latter may result from a fast selection of strains able to utilise certain contaminants or dissolved carbon released by decomposing phytoplankton (section 5.1). On the other hand, no effect on single microalgae (*P. subcapitata*, *C. reinhardtii* and *T. pseudonana*) was observed at AA-EQS, although MAC-EQS concentrations were able to inhibit PSII (section 5.2). Nevertheless, diatoms are known to absorb chemicals present in the environment which may lead to accumulation in higher organisms and enhance the toxicity (Carvalho et al. 2011).

At higher trophic levels, acute immobilisation of *Daphnia magna* along with toxicity and alterations in development of frog and fish embroys were among endpoints affected by concentrations close to EQS (section 5.3), which, however, are general and may result from exposure to multiple substance types. Embryotoxic and teratogenic compounds present in Mix14 and Mix19 act on developmental processes in a poorly understood way and their effects have been reported at concentrations exceeding those detected in surface waters (Fort et al. 1989 and 2004; Hatch and Burton,1; Bonfanti et al. 2004; Richards and Cole, 2006; Saili et al. 2013), therefore a possible synergetic/additive action can hardly explain the observed effects.

The scenario is more straightforward regarding endocrine disrupting chemicals, whose effects are only linked to molecules affecting the endocrine system and may be assessed by employing several *in vitro* and *in vivo* bioassays, some of which involving binding to specific molecular receptors. Estrogen-mediated responses to the reference mixtures were investigated for compounds belonging to different functional classes (synthetic steroid hormones, plasticizers, intermediates of industrial processes, antimicrobial agents) and able to mimic natural hormones with consequences on sexual functions and differentiation in aquatic organisms. These substances display varying affinity for the estrogen receptor (ER) translated into different potency of impact and together with other chemicals in the mixture may bind to the ER in agonist or antagonist manner. Furthermore, a high flexibility of the hERα ligand-binding domain to host a variety of structurally different molecules into its active site is well documented (Baker, 2011).

The exercise showed estrogenic effects at concentrations close to EQS, even though some assays were not sensitive enough to quantify responses below the limit of detection. Differences between estimated and experimental 17β-estradiol equivalent concentrations (EEQ) may result from antagonistic mixture effects (ER-CALUX and MELN assays) (Carvalho et al. 2014). The outcomes were confirmed by the upregulation of the ER-

mediated *cyp19a1b* expression in fish embryo brains during early and critical development stages.

Overall, selected bioassays employed in this exercise resulted suitable for detection of mixture effects in biota at concentrations believed environmentally safe and provide a promising alternative to chemical methods in the environmental assessment of surface waters. In particular, estrogenicity assays permit to overcome problems reported by Member States to quantify E2 and EE2 at the regulatory EQS values which fall below the limits of quantification (LOQ) of routinely used chemical analytical methods (Loos, 2012). It was further demonstrated by Kase et al. who tested five in vitro EBMs showing that these methods are less matrix-dependent and able to provide LOQ values below established effect-based trigger values (EBT) which are lower than those achieved by most commonly used chemical methods (Kase et al. 2018). Moreover, the results obtained with both approaches overlapped, indicating the EBT of 400 pg/L for mixture estrogenicity as suitable to distinguish between differentially polluted samples (Kase et al. 2018). Complementarity between bioassays covering several trophic levels and comparability of results among the laboratories lay a solid base for a future development of a monitoring platform as a holistic approach for water management linking chemical and ecological risk assessment.

7. CONCLUSIONS AND OUTLOOK

The present exercise highlights the gaps in current risk assessment under the WFD and MSFD in the context of chemical mixtures, unknown substances present in the environment including bioavailable metabolites and products of reactions between interacting componds, and mixture-related safety thresholds. Even though the majority of the bioassays did not measure an effect for the chemical mixtures tested when the single compounds were present at EQS equivalent concentrations, effects were visible for several biological endpoints. At increased concentrations (10-fold EQS-equivalent) of individual chemicals, biological responses were elicited in numerous bioassays. Both evidences prove the applicability of bioassays for the assessment of fresh and marine waters characterised by low concentrations of pollutants and of highly impacted wastewater treatment plant effluents.

A battery of bioassays covering the most relevant endpoints may in the first instance inform about types of ecotoxicological impact on biota taking into account combined action of substances in realistic samples. As demonstrated here, inhibition of growth and photosynthesis in algae may be an example of tests for contamination by herbicides and their additive effects in mixtures which would cover the impact of chemicals not considered in regulatory guidances. The most appropriate bioassays can be employed locally based on the identified contamination profiles (e.g. from households, industry, agriculture, hospitals) and concentration range applicability with regard to sentinel species intended as pollution-sensitive and key trophic organisms. In the same way, the composition of reference mixtures can be adapted in order to avoid false negative resuts when highly potent representative substances are not included.

While the chemical assessment employed to confirm the presence and determine the concentrations of single compounds lacks information on realistic effects in biota, ecological assessment focuses on alterations at population/community level which may depend on upstream changes in the food chain, therefore it identifies late adverse outcomes, often irreversible. Bioassays provide methods to bridge both approaches according to the precautionary principle in a way that early effects from a multitude of co-occurring substances can be timely detected.

The use of tailored reference materials with substances of known EQS enables a direct translation of outcomes into regulatory safety values "quantifying" toxicity also for mixture effects and would be of advantage for harmonisation of future regulatory applications. Further EU-wide exercise simulating assessment on real environmental samples is necessary to define bioassays, composition of reference mixtures and validate procedures with major benefits through intercalibration studies. Particular attention should be paid to sensitive estrogenicity assays capable of detecting responses to low (regulatory) concentrations of estrogenic compounds (E2 and EE2) and to other substances eliciting estrogenic effects in the abcence of hormones (e.g. 4-nonylphenol, bisphenol A, triclosan).

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List of abbreviations and definitions

AA-EQS	Annual Average Environmental Quality Standard
AESD	Analytical and Environmental Sciences Division, King's College London, UK
AhR	Arylhydrocarbon Receptor
AR	Androgen Receptor
BCP	Bacterial Carbon Production
BHAM	University of Birmingham, UK
BOKU,	University of Natural Resources and Life Sciences, Vienna
DEET	Diethyltoluamid
DEHP	Bis(2-ethylhexyl) phthalate
DEQ	Diuron equivalent
DHT	Dihydrotestosterone
DHT-EQ	Dehydrotestosterone equivalent
DNS	Department of Nutritional Sciences, King's College London, UK
DTU	Technical University of Denmark
EASZY	Endocrine Active Substances acting through ERs, using transgenic *cyp19a1b*-GFP zebrafish embryos
Eawag	Swiss Federal Institute for Environmental Science and Technology / ETH
ECOTOX	Oekotoxzentrum/Eawag, Dübendorf, Switzerland
EBM	Effect-based method
EBT	Effect-based trigger value
EDCs	Endocrine Disrupting Compounds
EEQ	Estrogen Equivalents
EQS	Environmental quality standard
EQSD	EQS Directive
EQS-eq	Environmental quality standard concentration equivalent
10xEQS	Ten-fold environmental quality standard concentration equivalent
ER	Estrogen Receptor
EROD	7-Ethoxyresorufin-O-deethylase
FET	Fish Embryo Toxicity test
FETAX	Frog Embryo Teratogenesis Assay
GABA	γ-Aminobutyric acid
GC	Gas chromatography
GFP	Green fluorescent protein
HUJI	The Hebrew University of Jerusalem, Israel
ICP	Inductive Coupled Plasma
INERIS	National Institute for Environmental Technology and Hazards, Verneuil en Halatte, France
IR	Induction ratio
ISPRA	Istituto Superiore per la Protezione e la Ricerca Ambientale, Rome, Italy
JRC	Joint Research Centre (European Commission)
LBD	Ligand binding domain
LC	Liquid Chromatography
LC-MS-MS	Liquid Chromatography (tandem) Triple Quadrupole Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
MBSS	Marine Biological Station Piran - National Institute of Biology, Slovenia
MS	Mass Spectrometry
MSFD	Marine Strategy Framework Directive
NGM	Nematode growth medium

NIFES	National Institute of Nutrition and Seafood Research, Bergen, Norway
NTNU	Norwegian University of Science & Technology (NTNU), Trondheim, NORWAY
ORU,	Life Science Center, Örebro University, Sweden
PPAR	Peroxisome Proliferator-Activated Receptor ɣ2
PXR	Pregnane X Receptor
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RECETOX	Research Centre for Toxic Compounds in the Environment, Masaryk University, Brno, Czech Republic
REF	Relative Enrichment Factor
REQ	Rosiglitazone Equivalent
RM	Reference Material
RTG-2	Rainbow Trout Gonad cell line
SPE	Solid-Phase Extraction
SRM	Selected reaction monitoring
TCDD	2,3,7,8-tetrachlordibenzodioxin
YES	Yeast estrogen screen
WFD	Water Framework Directive
UNIPMN	Università del Piemonte Orientale, Alessandria, Italy

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